A STUDY ON THE CORRELATION OF ADENOSINE DEAMINASE AND GLYCATED HAEMOGLOBIN IN THE PATIENTS OF TYPE 2 DIABETES MELLITUS.
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Abstract
Background & Objectives: Adenosine modulates insulin action on various tissues and its concentration in tissues is affected by Adenosine Deaminase (ADA) levels. ADA is an enzyme involved in purine metabolism and is considered to be a marker of T cell activation. Immunological disturbances in type 2 diabetic individuals have an association with cell mediated responses and inappropriate T-lymphocyte function. Hence, the study was undertaken to determine the levels of Serum ADA activity in patients of type 2 DM and its correlation with parameters of glycemic profile such as Fasting blood sugar (FBS) and Glycated Haemoglobin.

Material and Methods: A total of 100 patients diagnosed for type 2 DM visiting the Outpatient Department of General Medicine and Endocrinology at Mahatma Gandhi Medical College & Hospital, Jaipur were enrolled for the study based on predefined inclusion and exclusion criteria. Blood samples were collected for all enrolled patients and analysed for the investigations like Serum BSF, HbA1c and Serum ADA.

Results: In the study, BSF, mean HbA1c and serum ADA level was significantly higher in diabetic group in comparison to control group (p=0.000). The diabetic group was subdivided on the basis of HbA1c levels, HbA1c ≤ 8% as good glycemic control and HbA1c > 8% as poor glycemic control. BSF, mean HbA1c and serum ADA levels were observed to be significantly higher in poor glycemic control group as compared to that of good glycemic control. A significant positive correlation between S. ADA and HbA1c activity was also seen (r= 0.388).

Conclusion: Increased ADA level can be used to determine the glycemic status in the patients of type 2 DM and serve as a marker for insulin resistance. Hence, by analysing ADA levels in diabetes, glycemic control and insulin resistance can be assessed. Raised ADA levels can be an early indicator of progressive diabetic change and help to take preventive measures for the development of diabetic complication and thereby improving the outcome of the disease.

Keywords: Diabetes Mellitus, Adenosine Deaminase, Glycated haemoglobin

Introduction
The worldwide prevalence of diabetes has continued to increase dramatically.[¹] World Health Organization (WHO), shows that India is going to face a big challenge posed by the rising prevalence of diabetes and its complications, unless steps are taken to implement the primary and secondary prevention measures in diabetes.[²] According to the WHO in 2016, about 422 million people worldwide were living with diabetes[³] and International Diabetes Federation (IDF) estimated that the number of people worldwide affected with diabetes is expected to be around 438 million by 2030 and 642 million by 2040.[⁴] Indian Council of medical research estimated that the diabetes prevalence in individuals above 14 years was 2.1% in urban areas and 1.5% in rural areas while in those above 40 years of age, the prevalence was 5 per cent in urban and 2.8 per cent in rural areas in 1972-1975.[⁵]

Type 2 DM is a metabolic disorder characterized by the presence of chronic hyperglycemia with disturbances in the metabolism of carbohydrates, fats, and proteins.[¹] Long term hyperglycemia predisposes to long term micro and macro vascular complications.[⁵] The American Diabetes Association (ADA) includes the following criteria to be the investigative benchmarks:[⁷]
A fasting plasma glucose (FPG) level of 126 mg/dL (7.0 mmol/L) or more, or
A 2-hour plasma glucose level of 200 mg/dL (11.1 mmol/L) or more during a 75-g Oral glucose tolerance test (OGTT), or
Random plasma glucose of 200 mg/dL (11.1 mmol/L) or more in a patient with typical signs of hyperglycemia and hyperglycaemic emergency.

Serum Adenosine deaminase activity (ADA), an enzyme present in red cells and vessel wall catalyses the irreversible hydrolytic deamination of Adenosine to inosine and 2'-deoxyadenosine to 2'-deoxynosine which is then further converted to hypoxanthine, xanthine and finally to uric acid (UA). ADA activity is widely distributed in most organs like heart, skeletal muscle, liver, fatty tissues etc. In addition to this, Adenosine is responsible for increasing glucose uptake into cells. Thus, higher ADA activity in insulin sensitive tissue will decrease adenosine levels which in turn would decrease glucose uptake into cells.

Serum ADA plays an important role in maturation and activation of lymphocyte. ADA is associated with Thymocyte activity and high lymphocyte ADA activities were found in diseases with cell mediated immune response. Its blood levels may help in predicting immunological dysfunction associated with Type 2 DM. Chronic hyperglycemia leads to increased oxidative stress by forming enediol radicals and superoxide ions by NADPH oxidase system and increases ADA levels, both leading to insulin resistance.

Glycated haemoglobin (HbA1c) is a routinely used marker for long-term glycemic control. It is an indicator for the mean blood glucose level and predicts the risk of development of complications in diabetes patients. Apart from classical risk factors like dyslipidaemia, elevated HbA1c has now been regarded as an independent risk factor for cardiovascular disease in subjects with or without diabetes.

Materials & Methods:

The study was conducted in Department of Biochemistry in collaboration with the Department of General Medicine of Mahatma Gandhi Medical College & Hospital, Jaipur. Patients diagnosed with type 2 DM visiting the Outpatient Department of General Medicine & Endocrinology were enrolled for the study. The study was conducted after seeking approval from the Institutional Ethics Committee (IEC) informed consent was taken before enrolling the patients for the study.

The study subjects were divided into two groups.
1. Diabetic Patients (n= 100)
2. Control group (n= 50)

Inclusion Criteria
a) 20-65 years of age.
b) Known cases of type 2 diabetes mellitus
c) Patient willing to participate.

Exclusion Criteria
a) Patients not willing to participate.
b) Patients on insulin treatment, gestational diabetes mellitus, haemolytic anaemia, Hb variants.
c) Patients with chronic diseases such as tuberculosis, rheumatoid arthritis, gout, renal failure, immunological disorders which alters ADA level.
d) Pregnant & Lactating females.
e) Patients on any substance abuse.

Control group
A control group comprising 50 healthy non-diabetic subjects of comparable age and sex distribution were enrolled for comparative study. Detailed history, clinical examination and relevant investigations were conducted to exclude controls suffering from any such disease which is likely to affect serum ADA and blood glucose level.

Methodology

Detailed history and physical examination of the patients were done. Patients were asked to provide a detailed history and were subjected to a physical examination. An informed consent was taken before the collection of the sample from cases and controls. The control subjects had the same exclusion criteria as the cases and were not on any drug regimens which could influence the study. The study was conducted after approval from the institutional Ethics committee. Blood samples after overnight fasting were collected by standard aseptic techniques.

Plasma blood sugar in fasting sample, HbA1c, Serum ADA were estimated by colorimetric method on fully automated analyser VITROS 4600.

Following Parameters to be estimated
1) Blood Sugar Fasting (BSF)
2) Glycosylated haemoglobin (HbA1c)
3) Serum Adenosine Deaminase (ADA)

Estimation of Blood Glucose

Quantitative determination of Serum glucose was done by colorimetric –Glucose Oxidase Peroxidase method.
Reagents:
Slide ingredients are:
● Reactive ingredients per cm²
  - Glucose oxidase (Aspergillus Niger) Peroxidase (horseradish root); 1, 7-dihydroxynaphthalene (dye precursor) and 4-aminoantipyrine hydrochloride (dye precursor).
● Other ingredients: Pigment, binders, buffer, surfactants, stabilizers and cross-linking agent.

Principles
The VITROS GLU Slide method is performed using the VITROS GLU Slides and the VITROS Chemistry Products Calibrator Kit 1 on VITROS 250/350/950/5, 1 FS and 4600 Chemistry Systems and the VITROS 5600 Integrated System. The VITROS GLU Slide is a multilayered, analytical element coated on a polyester support. A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The oxidation of sample glucose is catalyzed by glucose oxidase to form hydrogen peroxide and gluconate. This reaction is followed by an oxidative coupling catalyzed by peroxidase in the presence of dye precursors to produce a dye. The intensity of the dye is measured by reflected light.

Table 1: Test Type and Conditions

| Test Type   | VITROS System | Approximate Incubation Time | Temperature | Wavelength | Reaction Sample Volume |
|-------------|---------------|-----------------------------|-------------|------------|------------------------|
| Colorimetric| 5600, 4600    | 5 minutes                   | 37°C (98.6°F) | 540 nm     | 10 µL                  |

Reaction Scheme

\[
\beta-D-glucose + O_2 + H_2O \rightarrow D-gluconic acid + H_2O_2 \\
2H_2O_2 + 4-aminoantipyrine \rightarrow \text{red dye 1,7dihydroxynaphthalen}
\]

Calibrators
VITROS Chemistry Products Calibrators Kit 1 was used for calibration.

Reference ranges

| Blood Glucose Levels     | Reference ranges |
|--------------------------|-------------------|
| Fasting blood glucose    | 60-100 mg/dl      |
| Post-prandial blood glucose | 80-140 mg/dl    |
| Random blood glucose     | 60-110 mg/dl      |

ESTIMATION OF HbA1c

Principles of the Procedure
The determination of % glycated hemoglobin (HbA1c) is performed using the VITROS Chemistry Products HbA1c Reagent Kit.

Whole blood samples are haemolysed on the VITROS 5, 1 FS/4600 Chemistry Systems and the VITROS 5600 Integrated System. Calibrators, controls and haemolysed whole blood samples are mixed with Reagent 1 containing anti-HbA1c antibody to form a soluble antigen-antibody complex. Hemoglobin in the haemolysed whole blood is converted with Reagent 1 to a hematin derivative that is measured biochromatically at 340 nm and 700 nm. Unbound anti-HbA1c antibody reacts with polyhapten (hexapeptide-glycan, A1c Reagent 2) to form an insoluble antibody-polyhapten immune complex, which is measured turbidimetrically at 340 nm. After a calibration has been performed for each reagent lot, the hemoglobin A1c and Hb concentrations in each unknown sample can be determined using the stored calibration curves and the measured absorbance obtained in the assay of the haemolysed sample.

%A1c
%A1c is a derived test calculated from the quantitative measurements of hemoglobin and hemoglobin A1c.

Test Type and Conditions

| Test Type | VITROS System | Approximate Incubation Time | Temperature | Wavelength | Reaction Sample Volume |
|-----------|---------------|-----------------------------|-------------|------------|------------------------|
| End-point | 5600, 4600, 5, 1 FS | 7 minutes                   | 37 °C (98.6 °F) | (HbA1c)340nm (Hb)340/700nm | 5.0µL                  |
Calibrators: VITROS Chemistry Products Calibrators Kit 31 was used for calibration.

Reagents

- **Reactive Ingredients**
  - HbA1c Reagent 1 (R1): HbA1c antibody (ovine serum) ≥0.5 mg/mL
  - HbA1c Reagent 2 (R2): HbA1c Polyhapten ≥8 μg/mL
  - DIL5 (R1): Tetradecyltrimethylammonium bromide (TTAB) <1% (w/v).

- **Other Ingredients**
  - HbA1c Reagent 1 (R1): Buffers, surfactant, stabilizers, and preservatives
  - HbA1c Reagent 2 (R2): Buffers, surfactant, stabilizers, and preservatives
  - DIL5 (R1): Surfactants, stabilizers, and preservatives

**Reagent Scheme**

| Specimen haemolysis | HbA1c Step 1: Immune Complex HbA1c & Hemoglobin Chromophore | HbA1c Step 2: Detect unbound (free) anti-HbA1c |
|---------------------|----------------------------------------------------------|--------------------------------------------------|
| Whole blood         | R1 (DIL5 Reagent 1) → Hemolyzing Reagent                 | Hemolysed whole blood                            |
| HbA1c Step 1: Immune Complex HbA1c & Hemoglobin Chromophore | Hemoglobin Chromophore, HbA1c-antiHbA1c immune complex and unbound anti-HbA1c (soluble) | HbA1c polyhapten/anti-HbA1c immune complex (turbid/insoluble) |
| Hemolysed whole blood | R1 (HbA1c Reagent 1) → Excess anti-HbA1c                |                                                  |
| HbA1c Step 2: Detect unbound (free) anti-HbA1c            | R2 (HbA1c Reagent 2) → Excessive HbA1c polyhapten       |                                                  |

**Reference Ranges**: HbA1c = 4.0-6.0%

**ESTIMATION OF SERUM ADA**

**Principle**

The ADA assay consists of four steps:

The ADA assay is based on the enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to UA and hydrogen peroxide (H₂O₂) by xanthine oxidase (XOD). H₂O₂ is further reacted with N-Ethyl-N-(2-hydroxy-3-sulphopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to generate quinone dye which is monitored in a kinetic manner.

**Reagent**

- Reagent 1: ADA R1 reagent
- Reagent 2: ADA R2 reagent

**Reaction Scheme**

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Adenosine + H₂O → Inosine + NH₃
Inosine + Pi → Hypoxanthine + Ribose-1-phosphate
Hypoxanthine +2H₂O + 2O₂ → Uric acid + 2H₂O₂
2H₂O₂ + 4AAP + EHSPT → 4H₂O₂ + Quinone dye
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Control: Adenosine Deaminase (Bovine Liver) and BSA.

**Reference Value**: Serum/Plasma = 4-22 U/L
Statistical Analysis

All results obtained were presented as mean ± SD in the patients as well as control group. The diabetic group was subdivided on the basis of HbA1c levels, HbA1c ≤ 8% as good glycemic control and HbA1c > 8% as poor glycemic control. The results were compared by applying Student’s t-test. The correlation of Serum ADA with BSF and HbA1c also calculated by applying Pearson’s correlation. P-value of ≤ 0.05 was considered as statistically significant.

RESULTS:

Table 1: represents the age distribution among control group and diabetic group. The mean age of subjects in the diabetic as well as control group was comparable (P=NS).

| Groups | No. of cases (N) | AGE (Years) (Mean ± SD) | t-value | P – value |
|--------|------------------|--------------------------|---------|-----------|
| Control | 50 | 47.60 ±13.17 | -1849 | NS |
| Diabetes | 100 | 51.12 ± 9.73 |

Table 2: represents the male: female ratio for all subjects of the study. The male: female distribution in control group was 56: 44 whereas in Diabetic group it was 67: 33.

| Groups | No. of cases (n) | Male | % | Female | % |
|--------|------------------|------|---|--------|---|
| Control | 50 | 28 | 56% | 22 | 44% |
| Diabetes | 100 | 67 | 67% | 33 | 33% |

Table 3.1: represents the mean BSF level in control group and diabetic group. There was significant difference in BSF level between control group and study group (P=0.000).

| Groups | No. of cases (n) | BSF (mg %) (Mean ± SD) | t-value | P – value |
|--------|------------------|--------------------------|---------|-----------|
| Control | 50 | 94.60 ± 12.79 | -10.73 | 0.000 |
| Diabetes | 100 | 261.68 ± 109.54 |

Table 3.2: represents the mean HbA1c levels in control group and diabetic group. The mean HbA1c levels in control group were 5.42±0.38 % and in diabetic group was 10.08 ± 2.46%. The difference between the two groups was statistically significant (P=0.000).

| Groups | No. of cases (n) | HbA1C (%) (Mean ± SD) | t-value | P – value |
|--------|------------------|------------------------|---------|-----------|
| Control | 50 | 5.42±0.38 | -4.29 | 0.000 |
| Diabetes | 100 | 10.08 ± 2.46 | 13.29 | 4 |

Table 3.3: represents the percentage of BSF in control and diabetic groups. The percentage of BSF in control group was 14% whereas in diabetic group it was 26%.
Table 3.3: represents the mean serum ADA levels in control group and diabetic group. The mean serum ADA levels in control group was 15.83 ± 4.35 U/L, while in Diabetic group it was 36.15 ± 17.47 U/L. Statistical analysis showed that the mean serum ADA level in diabetic group was significantly higher than the control group (P= 0.000).

Further, the diabetic patients (n = 100) were subgrouped on the basis of the HbA1c levels as:
- HbA1c ≤ 8% (Good Glycemic Control) n = 32
- HbA1c > 8% (Poor Glycemic Control) n = 68

| Groups | No. of cases (n) | ADA (U/L) (Mean ± SD) | t-value | P-value |
|--------|------------------|------------------------|---------|---------|
| Control | 50               | 15.83 ± 4.35           | -8.088  | 0.000   |
| Diabetics | 100             | 36.15 ± 17.47          |         |         |

Table 4: represents age distribution among two diabetic groups. The mean age for good glycemic control was 53.78 ± 8.48 years and for poor glycemic control was 50.85 ± 10.67 years.

| Groups | No. of cases (n) | Age (Years) (Mean ± SD) | t-value | P-value |
|--------|------------------|-------------------------|---------|---------|
| HbA1C ≤ 8.0 | 32            | 53.78 ± 8.48            | 1.363   | NS      |
| HbA1C > 8.0 | 68            | 50.85 ± 10.67           |         |         |

Table 6.1: represents the mean BSF level in two diabetic groups. The mean BSF level for diabetic group with good glycemic control was lower 224.75 ± 81.43 than that of diabetic group with poor glycemic control 276.97 ± 116.46 mg% and the difference was statistically significant (P=0.025).

| Groups | No. of cases (n) | BSF (mg %) (Mean ± SD) | t-value | P-value |
|--------|------------------|------------------------|---------|---------|
| HbA1C ≤ 8.0 | 32            | 224.75 ± 81.43         | -2.28   | 0.025   |
| HbA1C > 8.0 | 68            | 276.97 ± 116.46        | 4.12    | 0.000   |

Table 6.2: represents the mean HbA1c level in two diabetic groups. The mean HbA1c level for poor glycemic control was 11.68 ± 1.86 % and for good glycemic control was 7.31 ± 0.50 % and difference was significant (p=0.000).

| Groups | No. of cases (n) | HbA1c (%) (Mean ± SD) | t-value | P-value |
|--------|------------------|-----------------------|---------|---------|
| HbA1C ≤ 8.0 | 32            | 7.31 ± 0.50            | -12.14  | 0.000   |
| HbA1C > 8.0 | 68            | 11.38 ± 1.86           | 4.12    | 0.000   |

Table 6.3: represents serum ADA levels in two diabetic groups. The mean serum ADA levels was 28.68 ±9.33 U/L for good glycemic control and 41.58 ± 18.53 U/L for poor glycemic control and the difference was statistically significant (p=0.000).

| Groups | No. of cases (n) | ADA (U/L) (Mean ± SD) | t-value | P-value |
|--------|------------------|------------------------|---------|---------|
| HbA1C ≤ 8.0 | 32            | 28.68 ± 9.33           |         |         |
| HbA1C > 8.0 | 68            | 41.58 ± 18.53          |         |         |
Table 7: shows the correlation between Serum ADA, BSF, and HbA1c. When the comparison was made between serum ADA and HbA1c, there was a positive correlation (r=0.388) and the comparison was statistically significant (p= 0.000).

| Groups          | No. of cases (n) | ADA (U/L) (Mean ± SD) | t-Value | P-value |
|-----------------|------------------|------------------------|---------|---------|
| HbA1C ≤ 8.0     | 32               | 28.68 ± 9.33           | -3.716  | 0.000   |
| HbA1C > 8.0     | 68               | 41.58 ± 18.53          |         |         |

Discussion:

ADA plays a crucial role in lymphocyte proliferation and differentiation and its highest activity is seen in T-lymphocytes. High ADA activity might be due to abnormal T-lymphocyte responses or proliferation. In 1995, Reddy M et al. reported in their study that ADA activity in patients with Type 2 DM was higher as compared to the control group. Kurtil N. et al., 2004 reported in their study that serum ADA activity was higher in type 2 DM patients and correlated with HbA1c levels. They also suggested that Adenosine modulates the action of insulin on various tissues and its concentration in tissues is affected by ADA levels. ADA plays important role in insulin effect and glycemic control. Thus, depletion of adenosine due to increased adenosine deaminase activity would lead to insulin resistance in the body & subsequent hyperglycemia, which is a hallmark feature of diabetes mellitus. Hoshino T et al., 1994 also reported elevated ADA activity in the serum of Type 2 DM patients. In addition to this Amandeep Kaur et al., 2012 concluded that in type 2 DM patients there was a significant increase in serum ADA level with p value of <0.0001 when compared to controls. M Shivaparakash et al., 2006 observed significant increase in adenosine deaminase activity in diabetic patients and hypothesized that increased ADA activity may be due to altered immunity. In 2016, VijayAsamunneelsi et al., demonstrated that, serum ADA activity is increased in patients with type 2 diabetes mellitus. Mohammad Haghighatpanah et al., 2016 concluded that the mean BSF level was significantly (P< 0.001) elevated in patients with poor glycemic control (HbA1c>7%). The mean BSF level for diabetic group with good glycemic control was lower 224.75 ± 81.43 than that of diabetic group with poor glycemic control 276.97 ± 116.46 mg% and the difference was statistically significant (P=0.025). Khattab et al., 2010 reported that mean BSF levels was significantly elevated in patients with poor glycemic control. Serum ADA level had a significant positive correlation with FBS and HbA1c in type 2 diabetes mellitus patients. In accordance with our findings, Anjali C. Warrier et al., in 1995 showed in their study that increased serum ADA activity were correlated with Glycated hemoglobin and lipid peroxidation in DM patients. They suggested that decreased tissue adenosine levels is due to increase in ADA activity and is related to the severity of hyperglycemia and lipid peroxidation in diabetes mellitus. The enzyme ADA has a major role in purine metabolism and has also been identified as a reliable marker of cell mediated immune responses. However, its diagnostic importance has not been explored much. The present study reported a significant rise in serum ADA levels in type 2 DM patients and demonstrated a significant association of ADA with glycemic index (measured by HbA1c). This association suggests that estimation of serum ADA levels may have an important role as risk marker of CVD and other associated complications. The study, therefore, recommends further research on the importance of serum ADA estimation in type 2 DM and its association with other independent markers like lipid profile, CRP, homocysteine.

Conclusion:

The study was undertaken to determine the levels of Serum ADA activity in patients of type 2 DM and its correlation with parameters of glycemic profile such
as FBS and Glycated Hemoglobin. This association suggests that estimation of serum ADA levels may have an important role as risk marker of CVD and other associated complications. Increased ADA level can be used to determine the glycemic status in the patients of type 2 DM and serve as a marker for insulin resistance. Raised ADA levels can be an early indicator of progressive diabetic change and help to take preventive measures for the development of diabetic complication and thereby improving the outcome of the disease. The study recommends further research on the diagnostic importance of Serum ADA and its association with HbA1c & insulin resistance in larger cohort studies.

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