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RESEARCH ARTICLE

TESTING THE SURFACE FIXATION METHOD IN GESTATIONAL DIABETES MELLITUS

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ABSTRACT: **Introduction:** To test the surface fixation method contrasting urine samples of women with GDM vs healthy pregnant women. **Methods:** This was a pilot descriptive study. Three groups were conformed: A) Pregnant women with GDM, B) Women with healthy pregnancies and C) Non-pregnant healthy women. The positiveness of the surface fixation method was contrasted with Odds Ratio. **Results:** 12 women with GDM, 14 with healthy pregnancies and 9 non-pregnant women were included in the study.The OR for a positive surface fixation test when contrasting GDM vs Healthy pregnancies was of 2.7 while the value when contrasting GDM vs Healthy pregnancies + Non pregnant women was of 3.2 without reaching significant statistical difference in any case. **Conclusion:** the surface fixation method used with urine samples, suggests the existence of a transient antigen-antibody reaction that contributes to the inefficient insulin secretion.

KEY WORDS: gestational diabetes mellitus, immunodulation, surface fixation method.

INTRODUCTION:

Gestational diabetes mellitus (GDM) is still a clinical challenge around the world[1]. Inflammation contributes to its pathogenesis but the precise underlying mechanism remains to be explored[2]. Furthermore, autoimmunity is increasingly being recognized as a pathogenic component of GDM[3],although the identification of possible implicated autoantibodies is ongoing. For example, higher fT3 levels, potentially resulting from *de novo* synthesis or increased fT4 to fT3 conversion, may be an indicator of GDM risk starting early in pregnancy[4].

It is recognized that the action of autoimmune aspects in GDM are not solely restricted to autoantibodies.On the contrary, they include a miss-regulation of the Th1/Th2 equilibrium[5]. In this line of research IL-37 and 38 play important roles in autoimmunity, but their role in GDM development is unclear. The first may be protective, while the second, produced in the chorionic villi and umbilical cords, may be a response to local inflammation duringthe development of this pregnancy complication. Such a deregulated micro-environment may contribute to GDM development via an immune-mediated mechanism[6].

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Also concerning autoimmunity and GDM, evidence shows that in a number of women with GDM Zinc transporter 8 autoantibodies may be a marker for islet autoimmunity, but the clinical relevance of this finding requires further investigation[7]. Similarly, newer research underscores the importance of the maternal microbiome which may promote a pro-inflammatory environment conducive to autoimmune and metabolic disturbance[8].

Omics sciences using urine as a sample have developed year after year[9,10], but these advanced laboratory procedures are expensive and infeasible for developing countries. On the other hand, the surface fixation method developed by Ruiz Castañeda is based on immunoglobulins' property of adhering firmly to the filter paper on which the antigen-antibody reaction takes place[11]. This technique has been successfully tested in several diseases[12–14]. The aim of this study was to test the Ruiz Castañeda surface fixation method contrasting urine samples of women with GDM and healthy pregnant women.

MATERIAL AND METHODS:

Study participants

This was a pilot descriptive study. Pregnant women attendant at the Maternal-Fetal Service of the "Mónica Pretelini Sáenz" (HMPMPS), Health Institute of the State of Mexico (ISEM), Toluca, Mexico, were invited to take part. Three groups were formed: A) Pregnant women with GDM, B) Women with healthy pregnancies and C) Non-pregnant healthy women. GDM was diagnosed with a 75g oral glucose tolerance test. Women with multiple pregnancies, type 1 diabetes mellitus, glucose intolerance, and those with autoimmune or chronic diseases were excluded from the study.

Anthropometric Measurements

Weight (kg) was measured using a weight scale (SECA 711) and height (m) was measured using a mechanical column scale (SECA 220).With these two variables, the body mass index (BMI) (kg/m^2) was calculated.

Laboratory

A total of 3mL of venous blood (Vacutainer tubes) was taken from thefasting subjects. The concentrations of glucose (mg/dL), total cholesterol (mg/dL), and triglycerides (mg/dL) were determined by enzymatic methods (Atellica® Solution, Siemens-Healthineers) in the Clinical Laboratory of the HMPMPS. All measurements followed standardized procedures according to International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

Surface fixation method

In the Research Laboratory of the HMPMPS the urine sediment was obtained following these steps[15]:

1. In a 1-L bottle, 250ml of ethanol 96% was placed. Then, the patient was asked to collect the first morning urine for three days.
2. The container was left for 24 hours at room temperature to obtain a precipitate, and at the end of this time it was emptied, making a side hole to conserve the precipitate.

In the Research Laboratory of the Faculty of Chemistry, Autonomous University of the State of Mexico (UAEMéx), the surface fixation method was performed following these steps:

1. Once the sediment was obtained, it was centrifuged at 1500g for 3 minutes to separate the supernatant (Figure 1A).
2. The sediment was diluted with 0.9% saline solution (5mL). This step was repeated if there was too much sediment (Figure 1B).
3. The supernatant was placed in an Eppendorf tube so that the ethanol evaporated to the environment and the sediment was restored with 0.9% saline and stored (Figure 1C).
4. A solution of (0.2% bromophenol) in a 50-ml flask was prepared. 0.1g of bromophenol was added and up to 50ml was filled with distilled water (Figure 1D).

5. Another fixative solution was prepared in a 100-ml volumetric flask: 50ml of alcohol + 10ml of acetic acid + 40 ml of distilled water was added until the flask became warm (Figure 1D).
6. A drop of urine from each patient was added to the filter paper (Figure 1E).
7. The filter paper was taken to the stove for 5 minutes at 30°C (Figure 1F).
8. A drop of each patient's serum was placed on the drop of urine (Figure 1G).
9. The 0.2% bromophenol solution was spread on the filter paper containing the urine and serum drops (Figure 1H).
10. After waiting 1minute, the fixing solution was added to the same filter paper (Figure 1I).
11. The filter paper was dried at room temperature for one hour.
12. The last step was washing the filter paper with saline solution (Figure 1J). The results were observed and recorded. The urine of a patient with asthma was used as a positive control (Figure 2).

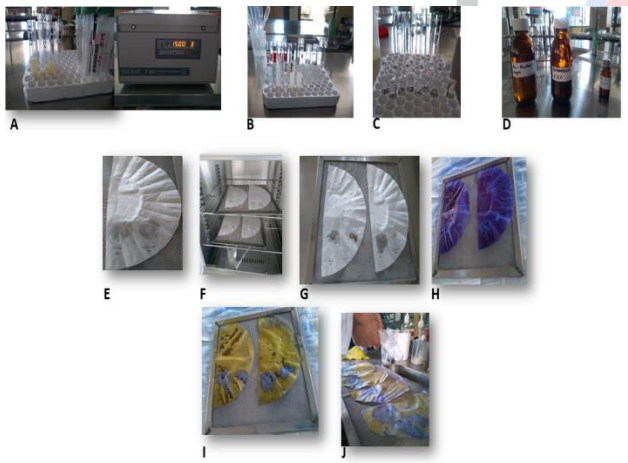

Figure 1.Surface fixation method in urine samples.
A) Sediment centrifugation.
B) Sediment diluted with 0.9% saline solution.
C) Supernatant evaporation and sediment is restored with 0.9% saline solution.
D) Bromophenol and fixative solutions.
E) Filter paper with a drop of urine.
F) Filter paper taken to the stove for 5 minutes at 30°C.
G) A drop of each patient's serum is placed on the drop of urine.
H) The 0.2% bromophenol solution is spread on the filter paper containing the urine and serum.
I) Fixing solution added to the filter paper.
J) Filter paper washing with saline solution.

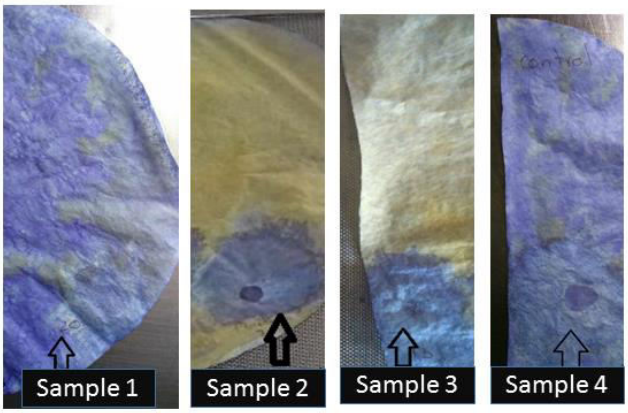

Figure 2.Sample report explanation
Sample 1: Healthy pregnant woman, Sample 2: Pregnant woman with Gestational Diabetes Mellitus, Sample 3: Non-pregnant healthy woman, Sample 4: Positive control.

Statistical Analyses

Age, anthropometric measurements and laboratorial tests were represented by measures of central tendency. First, the Kolmogorov test was performed to determine the normality of the variables. The one-way ANOVA test was used to contrast the variables among the three groups, and Student's T test or the Mann Whitney U test were used for multiple comparisons. These statistical analyses were carried out in the SPSS program, version 14. Odds Ratio (OR) was calculated for the positive surface fixation test and the diagnosis of GDM using MEDCALC[16].

Ethics

This study was approved by the Ethics and Research Committees of the HMPMPS (code 2017-06-532) and was performed in accordance with the ethical standards laid down in the updated Declaration of Helsinki, Fortaleza, Brazil, 2013. Informed consent was obtained from the patients.

RESULTS:

Table 1 General characteristics of the patients

Variable	Group			p
	GDM (N = 12)	Healthy pregnancies (N = 14)	Non pregnant women (N = 9)	
Age (years)	29.6 ± 6.9	27.9 ± 9.6	23.4 ± 5.9	0.034 [b]
BMI (kg/m^2)	26.1 ± 2.6	27.2 ± 4.1	22.3 ± 4.0	0.049 [b] 0.019 [c]
Cholesterol (mg/dL)	159.3 ± 39.3	222.3 ± 51.2	165.5 ± 34.9	0.003 [a] 0.013 [c]
Triglycerides (mg/dL)	198.2 ± 105.7	288 ± 111.4	110.8 ± 32.1	0.046 [a] 0.034 [b] ≤ 0.001 [c]
Glucose (mg/dL)	179.1 ± 43.6	83.6 ± 9.6	82.3 ± 4.4	≤ 0.001 [a] ≤ 0.001 [b]

BMI: Body Mass Index, GDM: Gestational Diabetes Mellitus.
a: between GDM and Healthy pregnancies.
b: between GDM and Non pregnant women.
c: between Healthy pregnancies and Non pregnant women.

Table 2 Odds Ratio (OR) for positive surface fixation test between GDM and Healthy pregnancies

Test	GDM	Healthypregnancies	OR (95% CI)	Zstatistic	p
Positive	10	9	2.7778 (0.4278 to 18.0386)	1.070	0.2845
Negative	2	5			

CI: Confidence Interval, GDM: Gestational Diabetes Mellitus, OR: Odds Ratio.

Table 3 Odds Ratio (OR) for positive surface fixation test between GDM and Healthy pregnancies + Non pregnant women

Test	GDM	Healthy pregnancies + Non pregnant women	OR (95% CI)	Zstatistic	p
Positive	10	14	3.2143 (0.5677 to 18.2004)	1.320	0.1869
Negative	2	9			

CI: Confidence Interval, GDM: Gestational Di

Table 1 shows the general characteristics of the three groups: 12 with GDM, 14 with healthy pregnancies and 9 non-pregnant women. As expected, there was a clear difference in the glucose value, but due to two outlier values in the GDM group the cholesterol values were lower in the GDM than in the non-pregnant women.

Table 2 shows the OR for positive surface fixation test when contrasting GDM and Healthy pregnancies and Table 3 shows the same between GDM and Healthy pregnancies + Non pregnant women. In the first, the OR was of 2.7 and in the second the OR was of 3.2 without reaching significant statistical difference in any case.

DISCUSSION:

Immunomodulatory treatment from urine created by Dr. Ruiz Castañeda has been widely used to treat different diseases. For those academics only now reading about Ruiz Castañeda's urine-based immunomodulation treatment, the process to obtain the preparation is as follows:

1. After obtaining the urine sediment as explained previously, it is centrifuged, evaporated, suspended in saline, filtered andthen diluted.
2. Sterility and culture tests are done to verify that the material is free of contaminants and can be used for treatment.
3. Finally, it is packaged under the strictest aseptic measures.

The instructions for the treatment are (using an ultrafine point insulin syringe with injections in the arms or abdomen):

1. Impregnation: Apply 10 units twice a week, increasing from ten units in ten-unit stepsuntil reaching 80 units.
2. Maintenance: When reaching 80 units the application changes toonce a week until the proper antigen is finished.

Although in clinical experience some patients improve, unfortunately there are no scientific publications to support these successful results. For that reason, our main effort was to confirm the isolation and bromophenol staining positiveness on the filter paper as it happened.

Based on our results it can be confirmed that a water-soluble substance was isolated from the urine of GDM patients which produced positive bromophenol staining. This preliminary report has been encouraging since all positive reactions were properly controlled with the serum of patients suffering from various clinical conditions.

Our study could be improved by perfecting the process conditions of the technique, both in spin time and heat exposure time. A limitation of this initial approach is the low number of patients and also, it has to be said, the non-specificity of the result due to the possibility of isolating more than one antigen-antibody reaction on the filter paper.

Urine samples from patients with GDM have been studied in several ways, for example, the group of Guo et al. using iTRAQ (the isobaric tags for relative and absolute quantification) for quantitative proteomics found 83 differential proteins increased and 36 proteins decreased in GDM. They concluded that the two candidate protein biomarkers (CD59 and IL1RA) in urine could be early, noninvasive diagnostic predictors of GDM[17]. More recently, López-Hernández et al. analyzed the urinary metabolome profile of GDM patients in the 3rd trimester of pregnancy based on liquid chromatography mass spectrometry, and identified 14 metabolites that were significantly up-regulated in the urine of GDM patients[18]. It is expected that using the above listed techniques it could be possible to identify, in a detailed way, the predominant molecules isolated through the surface fixation method for every disease in which the immunoglobulins' property of adhering firmly to the filter paper on which the antigen-antibody reaction takes place could be used.

In conclusion, the surface fixation method used with urine samples suggests, in the specific case of GDM, the existence of a transient antigen-antibody reaction that contributes to inefficient insulin secretion.

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