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Original Research Article

Presence of circulatory autoantibodies against glycated histones in diabetic patient in Saudi Arabia

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ABSTRACT

Background: Advanced glycation end products (AGEs) in diabetic patients can trigger several autoimmune responses. This article aims to assess the presence of circulating autoantibodies against glycated histones and their role in complications in diabetic patients in the Saudi population.
Methods: A total of one hundred twenty samples were collected from diabetic patients with different age groups and healthy individuals as control. All serum samples were collected from Prince Sultan Military Medical City (PSMMC) in Riyadh City in Saudi Arabia. Glycated H2A was prepared and characterized using different physiochemical techniques. Then, ELISA was performed to assess the presence of circulating autoantibodies against glycated histones in diabetic patients' samples compared with control healthy individuals in the Saudi population.
Results: The glycation of H2A under our experimental conditions appears to be completed in 14 days. also, our data showed high circulating autoantibodies were detected against glycated H2A in all diabetic patients' plasma with different dilutions. Remarkably, diabetic patients' group 1 (under 20 years old group) showed highly significant binding activity values in each dilution. However, diabetic patients in groups 2 and 3 showed less binding but still significant values when compared to control healthy individuals.
Conclusions: This finding provides novel perspectives into existing of circulating autoantibodies against glycated histones in diabetes patients in Saudi Arabia. Therefore, these circulating autoantibodies might be used as valuable tools for understanding the glycation mechanisms in diabetic patients in addition to providing diagnostic and prognostic knowledge. However, their roles in diabetic complications need further investigation.

Keywords: Diabetes mellitus, Hyperglycemia, Glycated histones, Advanced glycation end products, Circulating autoantibodies, Enzyme-linked immunosorbent assay

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder due to the lack of physiological function of the insulin hormone that induced hyperglycemia and dyslipidemia.[1] DM is a common chronic health problem that affects people around the world. The World Health Organization (WHO) estimated 1.5 million deaths caused by diabetes in 2019.[2] The prevalence of diabetes has significantly increased since the last decade, approximately 18% of Saudi adults have DM.[3] Hyperglycemia is the hallmark of DM. Long-term exposure to hyperglycemia leads to chronic complications. For instance, the development of macro vascular complications includes atherosclerosis, cardiovascular diseases, or microvascular complication

that leads to retinopathy, nephropathy, and neuropathy.[4] Numerous studies suggest that exposure to chronic hyperglycemia plays a significant role in the pathogenesis of diabetic complications and many chronic diseases.[5,6] The role of hyperglycemia in the molecular or cellular basis of long-term complications remains unclear. However, several theories have been devised, particularly one that has received considerable interest is the role of protein glycation in diabetic complications.[7,8] The biochemical pathways between chronic hyperglycemia and tissue damage are not completely understood. Negre-Salvayre et al illustrated that there is several evidence indicating that the initial cause of tissue damage happened in diabetes is hyperglycemia.[9,10]

Advanced glycation end products (AGEs) are the result of the non-enzymatic modifications of biological macromolecules through reducing sugar.[11] The AGEs accumulation in vivo is involved in secondary complications of diabetes mellitus.[12] In addition, dicarbonyl compounds are highly reactive and contribute to the pathogenesis of diabetic complications due to their modulating effect on the structure and function of intracellular proteins.[13,14] A recent study shows that AGEs can also result from dicarbonyl compounds produced from the process of autoxidation of sugars and some metabolic pathways.[15,16] Reactive dicarbonyl compounds also known as carbonyl stress, accumulated five to six times in diabetic complications.[17,18] α-oxoaldehydes such as 3 deoxyglucoses (3-DG), methylglyoxal (MGO), and glyoxal increase several folds in diabetes patients, with higher AGEs derived from α-oxoaldehydes seen in tissue proteins.[14,19] Protein AGEs formed by 3-DG cause the inactivation of major cellular proteins, which may result in apoptosis, necrosis, or arrest of cell growth.[20] Several proteins including elastin, collagen, lens crystalline, and histones are more susceptible to AGE modification action.[21] Histones, i.e., H2A, H2B, H3, and H4 are long-lived proteins that are primary components of eukaryotic chromatin structure.[22,23] Additionally, there is evidence that AGEs have antigenic properties which generated the hypothesis that continuous formation and accumulation of AGEs in diabetes patients can trigger autoimmune response.[24-27] Detection of AGEs reported in previous studies concerning different pathologies, the main effect of nonenzymatic glycation in disease and health has become an issue of great significance.[28] There hasn't been much research done in this area of study. This article aims to provide novel perspectives into the role of glycated histones in diabetes patients in Saudi Arabia by characterizing glycated histones H2A and explore the implications of circulating autoantibodies against glycated histone H2A in diabetes complications.

METHODS

Specimen

One hundred twenty samples of peripheral venous blood in the tube having Sodium Fluoride and Potassium Oxalate as an additive were collected. Samples were collected from diabetic patients with different age groups and healthy individuals as control. The samples were divided into four groups: 30 samples for each group. The Control group samples were collected from healthy individuals. The other three groups' samples were collected from diabetic patients and were classified according to their ages as follows: one group of patients less than 20 years old, the second group of patients between 20 to 45 years old, and the third group of the patients more than 45 years old. All serum samples were collected from Prince Sultan Military Medical City (PSMMC), The study was conducted at CAMS, and PSMMC and ethical committee approved it (CAMS 082-37/38 and HAP-01-R-015).

Antigen preparation

Deoxyribose modification of H2A

Three reactions in three polystyrene tubes were used, in the first reaction, 5 ml of 1X PBS and 5mg of histone from calf thymus (H2A) from Sigma (Product number. H 9250-100 mg) were mixed and used as a control. In the second reaction, 20 mg of 2 deoxy D ribose sugar taken from Sigma Aldrich (Cas number 533-67-5) and 10 mg of H2A were dissolved in 10 ml of PBS. In the third reaction, more Sugar was added to increase the glycation probability by elevating the glycemic status, 40 mg of 2 deoxy D ribose with 10 mg of H2A were dissolved in 10 ml of PBS. The three tubes were incubated at 37 °C for 14 days in a dark place to avoid direct light. Also, an extra tube was used with different incubation times. So, 10 mg of histone was added to 20 mg of 2 deoxy D ribose and 10 ml of PBS. this tube was incubated at 37 °C for 7 days with the same experimental conditions as the previous tubes.

Dialysis of prepared antigens

The dialysis tubes from Sigma were filled with the components of the second and third tubes that have 10 mg of histone (H2A) and different weighing of sugar (20 and 40 mg of D ribose) with 10 ml of PBS. The tubes were closed by a clip from 2 sides and put in a flask had 1 l of PBS. The component of the flask was mixed by using a magnetic stirrer, and the buffer was changed every 4 hours to remove the excess unbinding D ribose sugar. After dialysis, small aliquots from the two treated tubes were made and stored at -20 C.

UV Spectrophotometry

The absorption spectra (a spectrophotometer from Biochrom type Libra S 22 was used) for control and glycated H2A samples were measured in the range between 200-750 nm to evaluate the glycation modification in the histone molecule. After dialysis, spectrophotometer and fluorescence measurement were performed for all tubes, control, histones modified with 20 mg of D ribose/10 ml of PBS (20 mg/10 ml) and 40 mg of D ribose/10 ml of PBS (40 mg/10 ml). The spectra reading

for all samples were measured after 7 and 14 days of incubation at 37 C.

Fluorescence measurements

Fluorescence spectra reading for all samples were measured at the same time with spectrophotometry which assessed the change in histone because of incubation with D ribose. The formation of AGE-chromophores on histone was measured by AGE-type fluorescence at 320/380 ($\lambda_{ex}/\lambda_{em}$). The procedure was done using SpectraMax5 from molecular devices and data were analyzed using the SoftMax Pro 6.2.1 software.

Carbonate-bicarbonate buffer preparation PH 9.6, M (0.05)

Sodium carbonate solution was prepared by dissolving 5.29 gm of sodium carbonate (Na_2CO_3) and 4.2 gm of Sodium bicarbonate ($NaHCO_3$) in 1000 ml of DW. pH meter was used to adjust the pH of the buffer. The electrode was washed with distilled water and introduced into the prepared carbonate-bicarbonate buffer. pH of the solution was measured and adjusted to pH 9.6.

Enzyme-linked immunosorbent assay (ELISA)

To assess the presence of circulating autoantibodies against glycated histones in diabetic patients' samples compared with control samples, the ELISA was performed. Glycated H2A made as described above were used as antigen and were added to the patient's serum. This interaction was measured by using an ELISA reader (SFRI, Reader IRE 96) and the result of binding between Ag and Ab was read on an ELISA reader at 405 nm. ELISA was performed in 96 well plates using patient samples and control samples. ELISA was carried out on flat-bottom polystyrene modules. The micro titer wells were coated with 50 µl of a prepared antigen "glycated histone with 40 mg D ribose" and incubated overnight at 37°C, a blank well was included. After the washing, 20g of milk powder dissolved in 100 ml of 1X phosphate buffer saline and 50 µl of the mixture was added into the wells and incubated 15 min at 37°C, to prevent nonspecific binding. Then, the plate was washed 3 times with PBS, after each incubation to remove the excess unbound substance. Frozen patient plasma samples were thawed at room temperature for 30 min then serial dilution of serum sample was done as follows; 1:1, 1:100, 1:200, 1:400, and 1: 800. One-to-one dilution, 50 µl from the origin sample was added in the first well of micro titer plate without any dilution as a concentrated sample. Then, four test tubes were needed for each sample, the first tube contained 990 µl of PBS and 10 µl of the patient sample as a 1:100 dilution. For the second tube 500 µl was transferred from the mixture of the first tube and 500 ul of PBS was added to make a 1:200 dilution. For the third tube, 500 µl of PBS was added, and transferred 500 µl from the mixture of the second tube to had 1:400 dilution. The last tube had 500 µl of PBS and 500 µl from the mixture of the third tube to form a 1:800 dilution. Then transfer 50 µl of each test tube to the microtiter plate. The plate was incubated at 37 C for 1 hour and washed three times with buffer. Then, 1 µl Conjugated Monoclonal anti-human IgG alkaline phosphatase clone GG5 antibody (Sigma Aldrich Product Number A 2064-1 ml) was used with 999 µl of the carbonate-bicarbonate buffer then 50 µl of the mixture put to each well and incubated for 1 hour at 37°C. Followed by three times washing with buffer. pNPP (Ab 83369) was added to each well; 20 mg of pNPP substrate "p-nitrophenyl phosphate" tablet from Sigma was dissolved in 20 ml of carbonate-bicarbonate buffer then 50 µl of this mixture added to each well and incubated 25 min at 37 C, away from the light because of the substrate sensitivity to the light. To stop the reaction, 50 µl of stop reagent was added, or 50 µl of (1 M) NaOH was used. The absorbance readings were measured using an ELISA reader ("IRE 96 reader" SFRI) and the absorbance endpoint was 405 nm. The control samples were run the same as the patient samples, but the blank is done without adding antigen. The samples were run in duplicate (n=2) to confirm the results. Also, the sunrise device (TECAN) was used to double-check the absorbance and confirm the results.

Statistical analysis

The study was conducted during January-December 2022. The data were statistically analyzed using statistical package for the social sciences (SPSS) version 23 software (SPSS Inc., Chicago, IL, USA). The mean±standard deviation (SD) presents the quantitative variable values of ELISA for different concentrations. Kolmogorov-Smirnov test of normality was used to check if these variables follow the normal distribution. Non-parametric Kruskal-Wallis tests were used to compare ELISA values between the normal control group and all patient groups for different concentrations. Non-parametric Mann-Whitney tests were used to compare the quantitative variable of ELISA within the three diabetic groups. The data was statistically significant when the p value was less than 0.05.

RESULTS

Preparation and characterization of glycated histone

UV-visible characterization of glycated-H2A

The greatness of hyperchromicity that showed histone glycated was detected in with the highest D ribose concentration (40 mg) as compared to other variants with less concentration 20 mg of D ribose and control (native histone only no deoxy D ribose). Increasing the incubation time to 14 days showed the same result as shown in (Figure 1). This finding suggests that the binding between 2 deoxy D ribose and histone appears as maximum glycation when we increase the quantity of 2 deoxy D ribose. The mean and standard deviation were calculated for each group (Figure 2). A significant difference was seen between the control and the glycated histone with the highest

concentration 40 mg of deoxyribose. So, this confirmed the presence of the glycation process (Table 1).

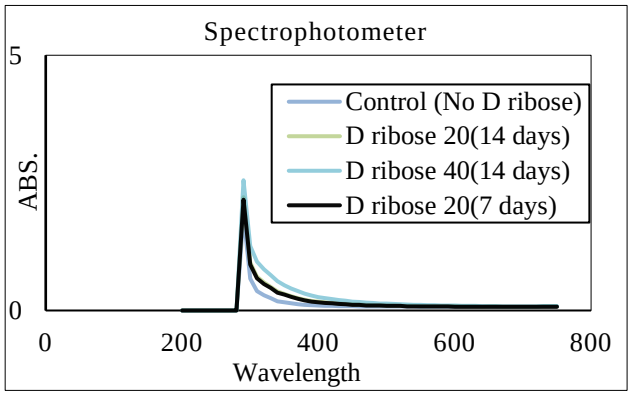

Figure 1: The scanned wavelengths of native and glycated histone showed maximum peak for glycated histone with 40 mg concentration of D ribose. The second-high peaks for glycated histone with 20 mg concentration of D ribose with different incubation days (7 and 14 days) and still showed the same elevation, and the lowest peak was detected in the control that has no D ribose.

Table 1: Descriptive analysis of spectrophotometry absorbance measurement of native histone, glycated H2A, and blank.

Parameters	Mean±SD
Blank	
PBS only	0.0±0.0
Native control	
5 mg H2A 5 ml PBS	0.10±0.14
Incubation for 14 days	
10 mg H2A 20 mg D ribose 10 ml PBS	0.18±0.33
10 mg H2A 40 mg D ribose 10 ml PBS	0.31±0.43
Incubation for 7 days	
10 mg H2A 20 mg D ribose 10 ml PBS	0.17±0.32

Fluorescence studies characterization of glycated-H2A

The formation of fluorogenic AGEs in deoxyribose-modified H2A samples was assessed from the fluorescence intensity (FI) of samples at 350 nm maximum emission detected. For the control (native H2A) no noticeable FI was detected at 300 nm. However, the histone (H2A) with D ribose at both concentrations (20 mg and 40 mg) gave approximately the same fluorescence intensity at the same wavelength as shown in Figure 3 (Figure showed result for 14 days' incubation only).

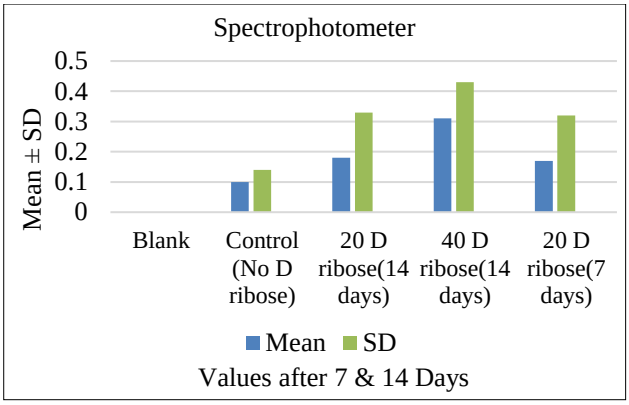

Figure 2: Descriptive analysis showed the mean and standard deviation for blank, control, glycated histone with 20 mg and 40 mg of 2 deoxy D ribose mixed with 10 ml of PBS in range between 200-750 nm.

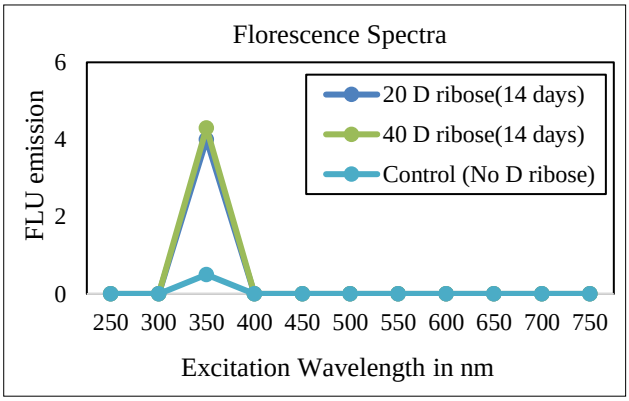

Figure 3: SpectraMax5 from molecular device used to detect the florescence spectra for incubated histone with 20 mg and 40 mg of 2 deoxy D ribose with buffer and read the highest emission at 350 nm.

High circulating autoantibodies were detected against glycated H2A in diabetic patients

The plasma of the patient and controls were examined to detect the presence of circulating autoantibodies against the prepared glycated H2A antigen in diabetic patients using indirect ELISA. A total of 120 serum samples were processed, 30 healthy serum samples as control and 90 diabetic serum samples divided into 3 groups according to age. ELISA was run twice for each sample for all groups with different plasma dilutions 1:1, 1:100, 1:200,1:400, and 1:800 as described in (Table 2).

ELISA reading indicated high binding activity between glycated histone (H2A) and the circulating autoantibodies with different patient plasma dilutions, screening a titer of at least 1:800 dilution was used as shown in (Figure 4). This finding suggests that glycated histone might be a potent immunogen and the binding between the glycated histones and the circulating autoantibodies could form an immune complex.

Table 2: ELISA reader at 405 nm for normal individuals versus diabetic patients with different groups under the same dilution concentrations.

Dilution	Control (normal individual) Sample #1	Group 1 (patient <20 years) Sample #14	Group 2 (patient 20-45 years) Sample #24	Group 3 (patient <45 years) Sample #26
1:1	0.142	0.734	0.242	0.294
1:100	0.134	0.830	0.252	0.345
1:200	0.136	0.736	0.251	0.423
1:400	0.156	0.827	0.266	0.486
1:800	0.149	0.86	0.311	0.387

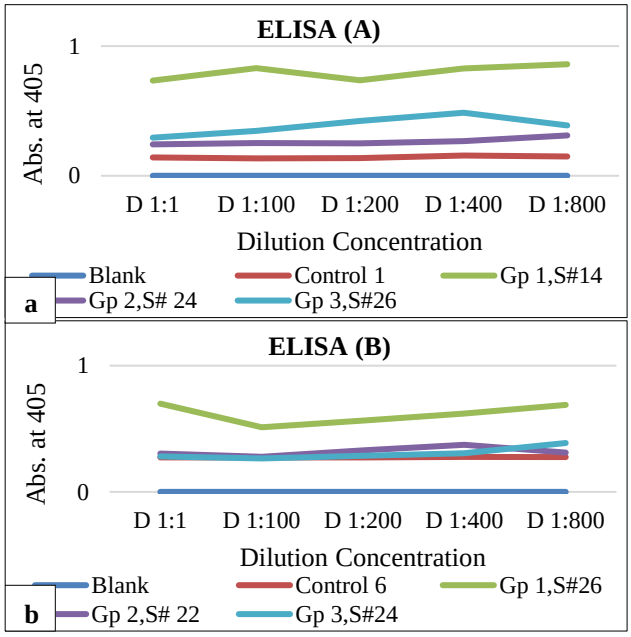

Figure 4: ELISA (a) and (b) illustrated the high binding between glycated H2A and circulating autoantibodies of histone in diabetic groups compared with blank and control.

All the 90 sera samples from all three groups showed high significant binding with glycated histone as compared to the control samples which showed no differences in absorbance in all dilution concentrations. Interestingly, patients in group one autoantibodies showed the highest binding activity with glycated histone (Figure 5). The comparative analysis of the data among the patient groups is shown in (Table 3). Kruskal-Wallis Test was used to find any significant results through different dilution concentrations (Table 4). Dilution 1:1,1:100,1:200,1:400 and 1:800 had highly significant results for all dilutions in diabetic groups which means there was significant binding activity between the glycated H2A and their circulating autoantibodies.

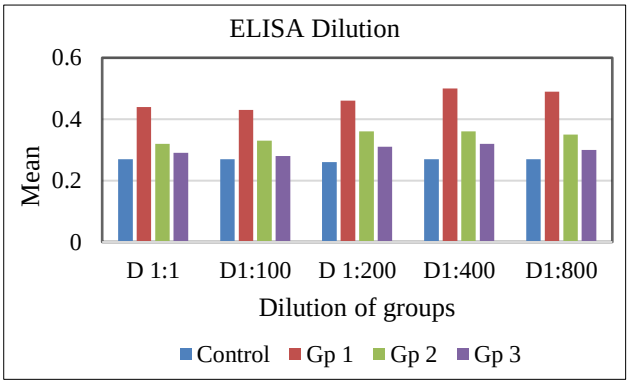

Figure 5: The average mean of dilution for each group, there is significant binding in the 3 patients' groups especially group 1; however, no significant differences in absorbance in control samples at all dilution concentrations.

Table 5 represented the values after applying the Mann-Whitney test to control and each diabetic group and made a comparison between different dilution values, group1 showed highly significant values in each dilution especially when compared with control. Groups 2 and 3 showed less binding but significant values when compared to the control. Mann Whitney test was used after the Kruskal-Wallis test when the latter test gives a significant result during comparing the parameters, but it can't give which group was significant. So, Mann Whitney can compare data for every 2 groups together to detect which group is statically significant.

Table 3: Statistical analysis using SPSS measured the average of the dilution for all groups and showed strong binding activity in diabetic group samples compared to control samples during the dilution process.

Descriptive analysis for, mean±SD	Dilution				
Classification	1:1	1:100	1:200	1:400	1:800
Control (age) (normal individual)	0.27±0.08	0.27±0.08	0.26±0.11	0.27±0.09	0.27±0.09
<20 (group 1)	0.44±0.16	0.43±0.16	0.46±0.19	0.50±0.23	0.49±0.23
20-45 (group 2)	0.32±0.15	0.33±0.13	0.36±0.15	0.36±0.13	0.35±0.13
>45 (group 3)	0.29±0.05	0.28±0.05	0.31±0.07	0.32±0.07	0.30±0.06

Table 4: Kruskal Wallis test showed a significant difference between all groups (normal and diabetic).

Dilution	1:1	1:100	1:200	1:400	1:800
Chi-square	27.3	22.7	17.1	15.2	22.5
Sig*	0.000*	0.000*	0.001*	0.002*	0.000*

*Significance probability.

Table 5: Mann Whitney test showed the highest statically significant binding activity in patients group 1.

Mann-Whitney U	1:1	1:100	1:200	1:400	1:800
Group 1 and 2	0.001*	0.005*	0.021*	0.025*	0.007*
Group 1 and 3	0.000*	0.000*	0.000*	0.000*	0.000*
Group 2 and 3	0.296	0.248	0.300	0.379	0.079

DISCUSSION

In this study, we prove the presence of circulating autoantibodies against the prepared glycated histones in diabetic patients as it is documented in earlier studies. Gugliucci's study, provides evidence for the in vitro formation of both pentosidine and other AGEs on histones.[29] The same group revealed three folds higher AGE levels in histones isolated from the liver of diabetic rats.[23] A similar result was also observed in diabetic patients.[30] The glycation of H2A under our experimental conditions appears to be completed by 14 days because further incubation did not yield much change in absorbance value. The percentage of hyperchromicity was increased at 290 nm when calculating the modified H2A with 40 mg and 20 mg of D ribose compared to the native histone by 26.6% and 15.8% respectively. The increase in Abs is indicative of glycation taking place that leads to AGE formation.[31] The hyperchromicity could be ascribed to the exposure of the chromophoric group or the generation of more chromophoric groups during the glycation process. Previous studies of increased absorbance in the range of 290–400 nm have been utilized to predict the formation of AGEs.[32] Deoxy ribose is the most reactive in the glycation of proteins.[33] Luciano et al incubated ribose with fetal calf serum to generate glycation products and found that glycated fetal calf serum is toxic to insulin-secreting cells and increases intracellular oxidative stress. Other studies on deoxyribose concluded the high reactivity of D ribose and its widespread use as a glycating reagent for research in AGE-related diseases.[34] The hyperchromicity observed in glycated H2A samples with deoxyribose at 290 nm could be due to changes in the microenvironment of UV-absorbing amino acid residues because of the glycation of lysyl/arginyl residues. Furthermore, changes in protein conformation have been attributed to the glycation-induced unfolding of proteins then, leading to its cross-linking, aggregation, and dysfunction.[35] The fluorescence measurement showed emission at 350 nm, it has been supposed that these fluorescence properties rose from pentosidine that was synthesized from deoxyribose glycation.[36] A fluorescence study was conducted to confirm the generation of fluorogenic AGEs. Previous studies have revealed that several fluorogenic AGEs, including pentosidine, are formed during the glycation process. An increase in the intensity of the fluorescence of the glycated entities around this excitation/emission wavelength may indicate the formation of pentosidine or other AGEs.[37] In general, fluorescence spectroscopy, and UV-visible spectroscopy can be used as preliminary tools for the detection of glycated products.[38]

To assess the relationship between the AGEs and different vascular complications associated with diabetes in the clinical samples, the ELISA technique was used. ELISA evaluated the degree of binding of serum autoantibodies in diabetes patients. The observed preference of antibodies in binding to modified H2A in comparison to its native counterpart demonstrates the specificity of the immune response against the glycated histone and is indicative of the presence of AGEs induced neo-epitope generation on the modified protein. The clinical studies reveal a higher recognition of glycated histone by circulating autoantibodies in patients with different types of diabetes. The appreciably higher binding of diabetic autoantibodies with glycated H2A as against its native counterpart in 90 out of 120 samples showed better recognition for the modified histone by diabetic autoantibodies. The expression levels of anti-H2A antibodies were found higher in the patients as compared to those against the native histone H2A (Figure 4). Dilution with 1:100,1:200,1:400 and 1:800 showed the formation of immunocomplexes between circulating autoantibodies in diabetic patient samples for the three groups and glycated histone embedded in the surface of the microtiter plate. This immune complex formation was confirmed by an increase in the absorbance reading in the diluted wells (Table 5).

The non-parametric analysis detected the significance of the patient sample groups. The average absorbances (M±SD) with the sera of diabetic patients binding to native histone and glycated histone were 0.26±0.09, 0.46±0.19, 0.34±0.14, and 0.30±0.06 respectively (Table 4). No significant binding was observed in healthy subjects in Control. Group 1 under 20 years old showed more binding and formation of immune complexes than the other two groups (Table 5).

Several studies detected antibodies against glycated H2A in a patient who has IDDM or DM with complications.[39,40] The majority of the glycated proteins in plasma exist as Amadori products rather than in the more labile Schiff base form and only a small part of the Amadori products undergo subsequent rearrangements to AGEs.[41] In our study, we incubate histone with 2 deoxy D ribose to make sure that the glycation process passes all steps to form AGEs with time. The possibility of reducing glycation and tissue AGEs or blocking RAGE opens the exciting prospect of delaying or preventing the onset of diabetic complications. Numerous compounds, both natural and pharmacological, are being investigated for their possible therapeutic potential.

Limitations

This study has some limitations which have to be taken into account while interpreting the results. The samples were collected from diabetic patients and healthy individuals who were conveniently selected and therefore might have introduced some selection bias, affecting the generalizability of the results. We took only 30 samples for each of the four groups and a larger sample size would have enhanced the statistical power and reliability of the findings.

CONCLUSION

In summary, this study has pointed out the general differences between the patient groups in the presence of circulating autoantibodies against modified histone. The overall results showed that all the diabetic patients at different ages may have circulating autoantibodies against glycated histone without knowing the level of glycation which could be not equal in each patient and might be strongly related to the duration and the type of diabetes mellitus. These circulating autoantibodies provide not only diagnostic and prognostic parameters to clinicians but also give valuable tools for a better understanding of glycation processes.[42] Additional data collection and an experimental approach are necessary to reveal the reasons behind the level of circulating autoantibodies against glycated histone and further research are required to elucidate the underlying mechanisms. This target can be used as a predictive biomarker for controlling disease complications.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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