

Genomic instability in patients with type 2 diabetes mellitus on hemodialysis

Roberta Passos Palazzo¹
 Pamela Brambilla Bagatini²
 Patrícia Brandt Schefer²
 Fabiana Michelsen de Andrade²
 Sharbel Weidner Maluf¹

¹ Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

² Instituto de Ciências da Saúde, Universidade Feevale, Novo Hamburgo, RS, Brazil

Objective: A previous study by our research group evaluated the levels of DNA damage using the comet assay in hemodialysis patients with type 2 diabetes mellitus. The same blood samples were also evaluated using the cytochalasin B micronucleus assay. A comparison of the results of the two assays is presented here.

Methods: Whole blood samples were collected from 22 type 2 diabetes mellitus patients on hemodialysis and from 22 control subjects. Samples were collected from patients early in the morning on Mondays, before the first weekly hemodialysis session. The cytokinesis-block micronucleus assay (CBMN) was used to evaluate genomic instability.

Results: The frequencies of micronuclei and nuclear buds were higher in patients than in controls (p -value = 0.001 and p -value < 0.001, respectively). There was a correlation between the frequency of micronuclei and DNA damage with the results of the comet assay (p -value < 0.001). The difference in the frequency of micronuclei and nuclear buds between patients and controls was more pronounced in the group with higher median comet values than in the group with lower comet values.

Conclusion: Our results suggest that the increased rates of DNA damage as measured by the comet assay and influenced by the weekly routine therapy of these patients has a mutagenic effect, thereby increasing the risk of cancer in this group.

Keywords: Diabetes Mellitus, type 2; Micronucleus tests; Comet assay; Genomic instability; Renal dialysis

Introduction

The prevalence of diabetes mellitus type 2 (DM2) is reaching epidemic proportions with an estimation of 366 million patients in 2030. Projections show that Brazil will continue among the top ten countries with the highest frequencies, possibly with as many as 11.3 million subjects with DM2 by 2030.⁽¹⁾ DM2 is a heterogeneous disorder characterized by different degrees of insulin resistance and defects in its secretion.⁽²⁾ This condition leads to a reduction in life expectancy and quality; chronic hyperglycemia increases the risk of heart disease, strokes, peripheral neuropathy, kidney disease, blindness and amputations.^(3,4)

Diabetes and hyperglycemia can be sources of DNA damage via the oxidation of DNA bases and sugar-phosphate binding sites.⁽⁵⁾ The occurrence of these alterations can result in mutagenic effects and/or DNA replication arrest and may be associated with risk for developing cancer in diabetes mellitus patients.^(6,7) The association between cancer and diabetes has been investigated extensively and most, but not all studies, found that DM is associated with an increased risk for several types of cancer.⁽⁸⁾

Lymphocytes are excellent markers of exposure because they circulate for years or even decades through different organs and accumulate DNA damage during their lifespan.^(9,10) Much evidence has been collected about the association between micronuclei (MN) induction and the development of cancer.⁽¹¹⁾

Our group recently published a study that assessed changes, over one week, in levels of DNA damage using the comet assay to evaluate hemodialysis patients with DM2. The results indicated a very significant weekly variation which is influenced by the accumulation of metabolites and the hemodialysis sessions.⁽¹²⁾ Although it was shown that these patients have increased DNA damage as identified by the comet assay, this damage may be transient as the test is unable to detect genomic instability after cellular division cycles.

The aim of this study was to evaluate the incorporation of genetic damage. The cytokinesis-blocked micronucleus (CBMN) test was used to measure the frequency of MN, nucleoplasmic bridges (NB) and nuclear buds and to compare the results with those of the comet assay in a group of DM2 patients on hemodialysis and controls.

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Corresponding author:

Fabiana Michelsen de Andrade
 Pró-Reitoria de Pesquisa e Inovação – PROPI
 Sala 201 F --Universidade Feevale
 R S 239, no. 2755, B. Vila Nova
 93352-000 – Novo Hamburgo, RS, Brazil
 Phone: 55 51 3586-8800 ext. 8938
 fabiana.andrade@feevale.br

www.rbhh.org or www.scielo.br/rbhh

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Methods

Whole blood samples were collected from 22 DM2 patients on hemodialysis at the Clínica do Rim in the municipality of Novo Hamburgo, southern Brazil. Table 1 shows the characteristics of the study sample. Hemodialysis sessions were carried out for 4-hour periods three times a week using cellulose diacetate dialyzers.

Patients were enrolled after signing a copy of the informed consent form signed by the main investigator by which general information about the study was provided in accordance with the guidelines of the Ethics Committee of the Universidade Feevale, Novo Hamburgo, RS, Brazil. In addition, all patients answered an individual health questionnaire as recommended by the International Commission for Protection against Environmental Mutagens and Carcinogens.⁽¹⁰⁾ The study was approved by the National Ethics Commission of Brazil.

All patients included in the study were receiving medications such as anti-hypertensive drugs (mainly angiotensin-converting enzyme inhibitors), blood sugar lowering agents and diuretics. In addition, infusions of human recombinant erythropoietin and iron hydroxide were administered. Just one patient reported a history of gastric cancer. Patients with chronic viral diseases (hepatitis, HIV) were excluded from the study.

A control group was composed of 22 healthy individuals matched in respect to age, gender and whether they were smokers. A comparison of the characteristics of patients and the control group is shown in Table 1.

Peripheral blood samples were collected from patients and control individuals early on Monday mornings before the first weekly hemodialysis sessions of the patients. For the CBMN assay, an aliquot of blood (0.5 mL) was added to 5 mL of RPMI 1640 medium supplemented with 20% fetal calf serum and 0.2% phytohemagglutinin. The culture flasks were incubated at 37°C for 44 hours after which 4.5-µg/mL

cytochalasin B was added. Twenty-eight hours later (72 hours of culture) the material was harvested according to the method described by Fenech & Morley⁽¹³⁾ and revised by Fenech & Crott⁽¹⁴⁾ and Fenech.⁽¹⁵⁾ The cell suspension was fixed in 3:1 methanol:acetic acid with no hypotonic treatment and dropped onto clean slides. The slides were then stained with Giemsa. Two thousand binucleate cells from each individual were scored for MN, dicentric bridges (NB between daughter nuclei),⁽¹⁵⁾ and nuclear buds (amplified DNA)⁽¹⁴⁾ (Figure 1) on slides identified by a code for blinded analysis.

The comet assay was performed according to a standard protocol of preparation and analysis.⁽¹⁶⁾ Slides were prepared by adding 300 µL of normal agarose solution to the microscope frosted slide and letting the agarose solidify (5-30 minutes at 4°C). A mixture of 5 µL whole blood with 95 µL 0.7% low-melting-point agarose was added to the slide and covered immediately with a coverslip. The slides were placed on a tray and kept in a refrigerator for 5 minutes until the agarose layer solidified. After that, the coverslip was gently slid off and the slide was lowered into cold, freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.2, to which 1% Triton X-100 and 10% DMSO had been added). After 4 hours at 4°C, the slides were gently removed from the lysing solution and the excess liquid was blotted away from the back and edges. The slides were placed in the horizontal gel box at 4°C which was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). The slides immersed in the liquid were left in the tray for 20 minutes before the power was turned on. Electrophoresis was performed at 25 V and 300 mA (~0.95 V/cm) for 20 minutes. The steps above were carried out under red light to avoid the induction of DNA damage. After electrophoresis, the slides were gently removed from the tank and neutralizing buffer (0.4 M Tris, pH 7.5) was added to the slides dropwise three times, letting them sit for 5 minutes each time. The slides were rinsed 3 times with distilled water, air dried for at

Table 1 - Sample characteristics

| | Patients | Controls | p-value |
|--|-----------------------|------------|---------|
| Sample size | 22 | 22 | |
| Age mean ± SD | 62.5 ± 9.4 | 63.0 ± 8.0 | 0.85 |
| Males - n (%) | 12 (54.0) | 5 (22.7) | 0.06 |
| Smokers - n (%) | 1 (4.5) | 3 (10.3) | 0.13 |
| Former smokers - n (%) | 3 (13.6) | 0 (0) | |
| Treatment duration in months - median (95% CI) | 18.0 (12.9 - 37.1) | - | |
| Fasting glucose in mg/dL - median (95% CI) | 240.0 (164.8 - 289.7) | - | |
| Urea before HD in mg/dL - median (95% CI) | 167.0 (149.9 - 190.3) | - | |
| Urea after HD in mg/dL - median (95% CI) | 36.0 (31.9 - 51.6) | - | |
| Ferritin in ng/mL - median (95% CI) | 244.3 (140.3 - 519.7) | - | |
| Iron in µg/dL - median (95% CI) | 53.3 (6.42 - 103.2) | - | |

SD: Standard deviation; 95% CI: 95% Confidence interval; HD: Hemodialysis

Reference values - fasting glucose: 60-110 mg/dL; blood urea: 10-50 mg/dL; ferritin: 30-323 ng/dL; iron: 35-150 µg/dL

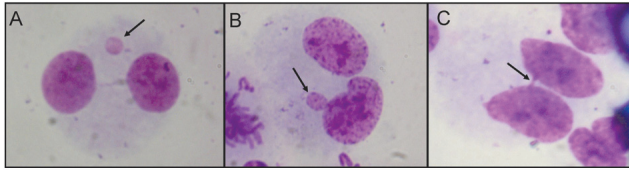


Figure 1 – Nuclear abnormalities detected by micronuclei test (arrows). A) micronucleus; B) nuclear bud; C) nucleoplasmic bridge

least 24 hours, and then fixed and stained with silver stain according to Nadin et al.⁽¹⁷⁾ To evaluate DNA damage, 100 cells per individual were analyzed under an optical microscope at 200x magnification. The cells were scored visually according to tail intensity into five classes, from undamaged (0) to maximally damaged (4).^(16,18) Therefore, in theory the total score for each individual ranged from 0 (all undamaged) to 400 (all maximally damaged). Slides from patients and controls were processed, coded, mixed and evaluated together. Differences between values for control individuals and patients were assessed using the Mann-Whitney test. Correlations between the various parameters were calculated using the Spearman correlation coefficient. The sample was divided according to the median of the range of comet assay results: ≤ 8 (18 individuals) or > 8 (17 individuals). The same damage parameters used in the CBMN test were compared for patients and controls in these two subgroups using the Mann-Whitney test. The software, SPSS 16.0 for Windows, was used for all statistical analyses.

Results

The variables in Table 1 were analyzed according to the parameters of DNA damage under study. No effects of treatment duration were found up to the time of sample collection; levels of glucose, urea, ferritin and iron were not significantly correlated with the number of binucleate cells with MN, nuclear buds and NB. Similarly, the frequency of

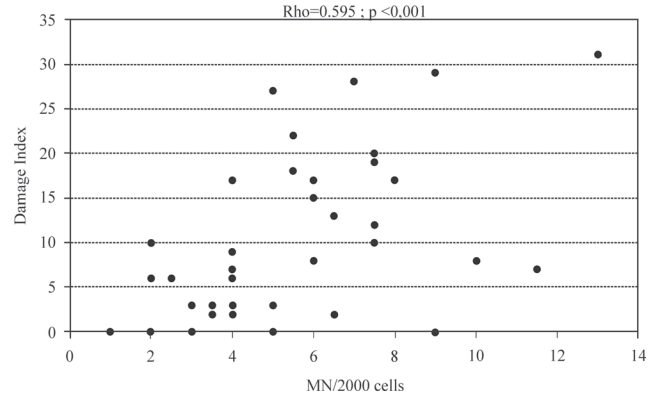


Figure 2 – Association between comet values and frequency of micronuclei in total sample (individual values)

iron (III) hydroxide intake and the dose of epoetin alpha used were not associated with DNA damage.

No effect of age on DNA damage was found. Only the association of male gender and increased number of nuclear buds was significant (p-value = 0.028). Comet assay values were significantly correlated with MN frequency (p-value < 0.001) but the correlation was not strong (rho = 0.595) (Figure 2).

The comparison of the control group and DM2 patients on hemodialysis revealed differences between the numbers of MN, nuclear buds and the comet assay results (Figure 3): significantly higher values were found for patients than for controls (p-value = 0.001, p-value < 0.001 and p-value = 0.049, respectively).

As the parameter evaluated by comet assay is a measure of DNA damage that can still be repaired after cell division and the micronuclei frequency measures fixed DNA damage, we hypothesized that MN values may be higher in only the patient group that had high DNA damage as seen by comet assay. If this hypothesis were correct, it would show that the detection of high damage index by the comet

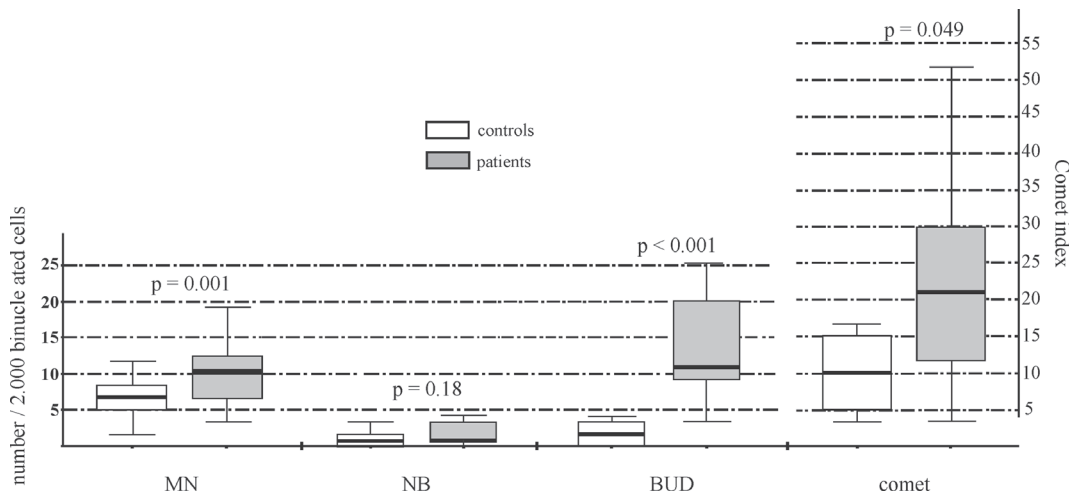


Figure 3 – DNA damage in peripheral blood cells of patients and controls. Internal horizontal lines in each group represent the median; boxes and whiskers represent interquartiles and lowest/highest ranges of individual values

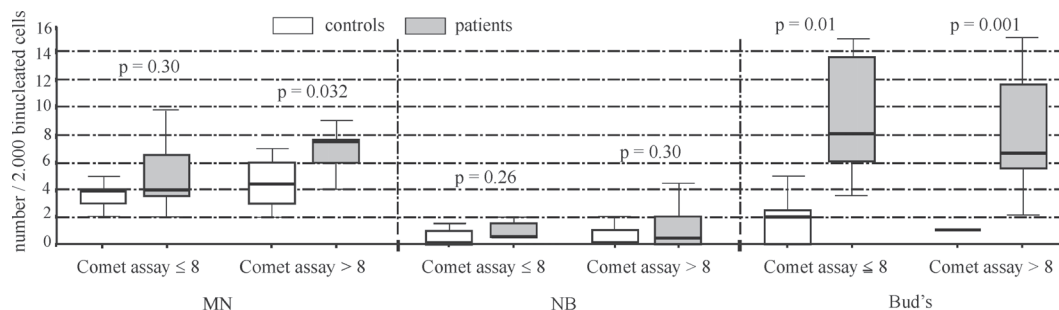


Figure 4 – Number of micronuclei, nucleoplasmic bridges and buds in patients and controls according to the median damage index of comet assay. Internal horizontal lines in each group represent the median; boxes and whiskers represent interquartiles and lowest/highest ranges of individual values

assay in this kind of patients is an indication of permanent DNA damage. Therefore, to test if the difference of CBMN values between patients and controls was modulated by DNA damage as scored by the comet assay the sample was divided according to the median comet assay results, which in our sample ranged from 2 to 31. The comparisons of CBMN parameters were repeated for patients and controls with lower damage (comet ≤ 8) and higher damage (comet > 8). Figure 4 shows the values of MN, NB and nuclear buds for the subgroups in the comparison of patients and controls. The difference in MN values between patients and controls was significant only in the group with higher comet values (p-value = 0.032), whereas in the group with lower comet values this effect was not statistically significant. Moreover, the difference in nuclear bud frequency was greater between patients and controls in the subgroup of individuals with higher comet values (p-value = 0.001) than in the subgroup with comet values below the median (p-value = 0.01). The number of nucleoplasmic bridges (NB) was not significantly different between patients and controls.

The same approach was used to investigate if values of MN, NB and nuclear buds are modulated by age and gender. Tests were performed according to the median age (in subgroups with ≤ 61 and ≥ 62 years), as well as according to gender, but no statistically significant differences were found. The association with smoking was not tested because of the small number of smokers and former smokers.

Discussion

Diabetic nephropathy is the most frequent isolated condition in patients with end-stage renal failure in Western countries.⁽¹⁹⁾ A recent study conducted by our research team⁽¹²⁾ found that two factors play an important role in the increase of DNA damage in hemodialysis patients with DM2: the disease itself, which leads to the accumulation of metabolites that damage DNA and hemodialysis, which removes metabolites but also increases the level of DNA damage in filtered cells. Thus, the amount of DNA damage in hemodialysis patients varies substantially during one week. The present study used the CBMN test to check how much of this damage is not repaired and, therefore, increases

genomic instability and the risk of developing secondary diseases, such as cancer.

The increase in MN frequency may show that not all damage caused by the accumulation of metabolites and blood filtration during hemodialysis is properly repaired. The analysis of MN in peripheral blood lymphocytes has been extensively used as a marker of chromosome loss and misrepaired DNA damage. In this study, there was a statistically significant correlation between MN frequency and DNA damage according to the comet assay results. This finding suggests that the greater the damage, the higher the number of lesions to DNA that may have been misrepaired. To confirm this hypothesis, the sample was divided at the median of comet assay values and a significant difference in MN frequency between patients and controls was only found in the group with higher values.

The NB frequency provides a measure of chromosome rearrangement.⁽²⁰⁾ The difference in NB frequency between patients and controls was not statistically significant, probably because of its low frequency and high standard deviations.

On the other hand, the number of nuclear buds was higher in patients, in spite of the great variation; this might be explained by the widespread use of drugs administered to patients. These drugs may be causing an amplification of genes related to cell intoxication. This amplified DNA moves to the nucleus periphery, forming buds.^(14,21)

The level of serum glucose of DM2 patients is different to the general population. Suba & Ujjal⁽²²⁾ conducted a study with tumor cells and found that increases in glucose levels intensifies DNA synthesis and increases free radical release, which may destroy DNA as well as the enzymes that take part in the repair process. The level of reactive species is elevated because of the suppression of reduced glutathione (GSH) synthesis in cases of hyperglycemia.⁽²³⁾ GSH is one of the most important agents in the antioxidative defense system of the cell, and its detoxifying effect reaches reactive agents before they can cause lesions.

Müller et al.⁽²⁴⁾ found that the effects of hemodialysis, and the types of dialyser and iron infusions used were associated with oxidative stress in uremic patients. They also suggested that the signs of oxidative damage might be

explained by exposure to the hemodialysis membranes which results in bioincompatibility that may activate the complement system, as well as white cells. Such an event promotes the degranulation and activation of adhesion molecules, a process that generates reactive oxygen species (ROS). These unstable species are responsible for several processes; one of them, lipid peroxidation, leads, once more, to the consumption of GSH, which decreases the power of the antioxidant defense system.

In addition to the events already described, hemodialysis patients often have iron-deficiency anemia, which may be explained by problems of erythropoiesis caused by chronic renal failure or to constant blood losses during each dialysis session.⁽²⁴⁾ To correct this imbalance, iron infusions are administered to patients, but studies show that these infusions may increase DNA damage.⁽²⁵⁾

Conclusions

In this study, there was a statistically significant correlation between DNA damage and the frequency of MN in the group with the highest values for comet assay results. This finding suggests that the greater the damage, the higher the number of lesions that remain after the action of the repair system. As the CBMN test assesses damage that remains after the action of the repair system, our results suggest that the increased rates of DNA damage measured by the comet assay of these patients have a mutagenic effect.

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