Genotoxicity evaluation in renal chronic patients undergoing hemodialysis and peritoneal dialysis through the micronucleus test

J.M. Roth[1], G.L. Garcias[2, 3], M.G. Martino-Roth[2], R.G. Restani[2], T.T.S. Gonçalves[2], S.L.S.Sphor[2], A.B. Ness[2].

[1]Mestranda em Saúde e Comportamento, Universidade Católica de Pelotas, RS, Brasil.
[2]Laboratório de Genética, Escola de Medicina, Universidade Católica de Pelotas, RS, Brasil.
[3]Departamento de Zoologia e Genética, Instituto de Biologia, Universidade Federal de Pelotas, RS, Brasil.

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* Corresponding author: Juliana Martino Roth.
Mailing address: Rua Visconde de São Gabriel, 131, Bairro Areal, Pelotas-RS, Brasil 96077-260 . e-mail: juroth@terra.com.br, fone:053 – 32286933.

Abstract

Patients with renal chronic disease have an increased incidence of cancer. It is well known that long periods of hemodialysis treatment are linked to DNA damage due to the oxidative stress. This genotoxic effect may cause loss of chromosome fragments, or even entire chromosomes, which form micronucleus (MN) after cell division, and can be detected by the micronucleus test. In the present case-control study we evaluated the genotoxic effect of hemodialysis treatment in 20 patients undergoing hemodialysis (HD), and 20 subjected to peritoneal dialysis (DP), matched for sex and age with 40 controls. Genetic damage was assessed by examining the frequency of micronuclei in 2,000 exfoliated buccal cells per individual. Our results revealed that patients undergoing hemodialysis treatment have a significantly higher frequency of micronucleated cells (5.60±5.31 MNC) compared to control subjects (1.50±2.01 MNC, $p<0.01$). Interestingly, the same was not observed for the peritoneal dialysis patients, who presented no significant differences in MNC (2.85±2.96) frequency compared to control individuals (3.25±3.85). In addition, we evaluated the possible association between creatine levels, smoking, alcoholic intake, age, time of treatment, incomes of the individuals (separately analyzed according to their gender) and the frequency of micronuclei. The results reported here indicate that the period of treatment is the only factor associated with increased MNC frequency among HD patients (Spearman coefficient 0.414, $p=0.01$). The number of MN cells found in individuals under six years or less of treatment was significantly lower (2.91±2.74 MN) compared to patients under seven or more years of treatment (8.89±5.96 MN, $p<0.05$). Overall, peritoneal dialysis may be a safer choice of treatment, but further studies need to be performed to investigate the risks and benefits of both treatments.

Introduction

In Brazil, according to the Brazilian Nephrology Society (SBN) census in 2002, from a total of patients undergoing renal substitution therapy, 54.523 (89.6%) remained in hemodialysis. Only 7.61%, from the total, were not kept directly by the Unique Health System. From 1999 to 2002 there was an increase of 27.7% in the number of clients. Our rates of prevalence of terminal renal chronic insufficiency (IRC) treated are about 4 times lower than in the USA and Japan and half the rates in Italy, France and Germany (Ajzen *et al*., 2002).

Barros *et al.* (1999) describes the IRC as a progressive loss of the renal function of depuration, which means glomerular filtration. Independent of the cause, IRC results from the irreversible loss of a high number of functional nephrons (Guyton *et al*., 2002). IRC is associated with the immunoinflammatory multifatorial syndrome which occurs precociously during the disease, getting worse as it progresses (Kan *et al*., 2002). In 1996/97, the main diseases reported as causes of terminal IRC were arterial hypertension (24%), glomerulonephritis (24%) and diabetes mellitus (17%) (Ajzen *et al*., 2002).

The hemodialysis (HD) used to compensate the deficient renal function in uremic patients can be harmful to the blood cells and there is a consensus that this type of patient has a high risk of oxidative stress and, as a consequence, damage to the DNA. Markert *et al.* (1988) suggest that, in patients kept in HD treatment, the blood interacts with biocompatible membranes where the circling neutrophyles use oxygen and generate reactive species of oxygen such as super oxide which leads to an increase of the inflammatory state. Ross *et al*. (1997) say that it is probable that the dialysis mechanism acts on the enzymes related to the glutation, removing substrates and acting factors.

In the dialysis process, one of the possible consequences is oxidative stress, which can result in damage to the DNA, including mutation of points by basis oxidation, breaks of simple and double chain, genomic instability and inhibition of the repair mechanism (Stopper *et al.,* 2004; Tarng *et al.,* 2002). These breaks can lead to the formation of fragments or losses of whole chromosomes that are not correctly distributed along the mitosis process, and in the next interphase these structures can be detected under the form of micronucleus (MN) (Stopper *et al*., 1999). The MN frequency, the comet assay in peripheral lymphocytes, as well as 8-hydroxy 2'-deoxyguanosine (8-OH-dG) found in the leukocytes, DNA mitochondrial in skeletal muscle and capillary follicles have been used as

biomarkers of damage in the DNA of chronic renal patients. According to Stopper *et al.* (2004) the alterations in the DNA can induce the carcinogenesis mechanism, being related to the aging process, neurodegenerative diseases, diabetes and arteriosclerosis.

In peritoneal dialysis (DP), the introduction and maintenance of a strange body (flexible catheter) in the organism and the distention of the abdominal cavity, periodical or permanent by the dialysis liquid (sometimes with high osmolarity) and the use of the peritoneum for the water and solute transport can generate infectious, mechanical and metabolite complications (Riella, 1996). Ha *et al*. (2000) have shown *in vitro* that solutions of DP with low pH or high levels of products of glucose metabolism or both promote in the human peritoneum the death of mesothelial cells and DNA damage. Gotloiob *et al.* (2003) exposing mesothelial cells of rats in high glucose concentrations that are more biocompatible, for 2h *in vivo,* have found an acceleration of the life cycle of the cells and in 30 days of culture fewer cells were left with nuclear forms and uncommon mitosis due to DNA damage. In agreement with the study of Wieczorowska *et al*. (2001) who used a DP solution with physiological pH and low levels of glucose products in the peritoneum of rats for 6 weeks, there was a fall in the intraperitoneal inflammatory process and peritoneal fibrosis ($p<0,005$), when compared to a DP solution with low pH and high levels of glucose products.

The mutagenic and carcinogenic effect of genotoxic agents in human populations exposed to the work environment accidentally, by medical treatment or by their lifestyle, has increased considerably. In hospital routines, many mutagenic agents are used for maintenance or diagnosis and treatment of patients. For most of the patients the benefit is obvious but, however, for some this benefit is only partial (Maluf e Erdtmann, 2000).

A way of studying the genotoxic effects in a population is to conduct monitoring studies, using pertinent biological parameters with short term manifestation and with micronucleus rehearsals which can identify damages in the DNA and/or in the chromosomes resultant from the exposure. The information obtained can be used as a precocious warning of the potential risk of developing long term health problems (Au, 1991).

Nowadays there are many tests, with *in vivo* models as well as *in vitro,* so that the test organisms varies from virus, bacterium, fungus, plants and insects to mammals, including humans (De Flora, 1998). Among the tests which analyze chromosomal mutations in

mammals, the micronucleus test stands out, as it is a simple and quick test which detects chromosomal breaks and losses (Hayashi *et al.*, 1994).

A high frequency of cells with micronucleus has been found by some researchers in chronic renal patients who have been undergoing hemodialysis for a long time (Stopper *et al.*, 1999). Kan *et al*. (2002) carried out an evaluation of the genotoxicity through the comet assay in chronic renal patients in HD programs and have found a significant DNA damage increase in comparison with the control group ($P<0.001$). These results agree with the ones from other studies, such as the one from Stopper *et al*. (2001) that also used the comet assay and have found significant DNA damage increase in the lymphocytes of 23 patients with chronic renal disease, suggesting that there is a relation between the hemodialysis treatment time and the damage caused in the DNA of these patients.

This study aims to compare the frequency of micronucleus and other alterations such as broken egg nucleus and binuclear cells to the cells of patients with chronic renal disease, in hemodialysis treatment and peritoneal dialysis and two control groups, aiming to detect if there is or not any genotoxic effect related to these treatments.

Material and Method

The current study was developed in the Genetic Laboratory of the Catholic University of Pelotas. The sample was made up of 80 people, including 20 patients with IRC in HD, 20 patients in DP, who formed the exposure group, and a control group made up of 40 healthy people without known exposure to genotoxic factors. The sample was hospitalized in the Santa Casa de Misericórdia of Pelotas Hospital, to undergo elective surgery and be matched for age and sex. Half of each group was male and the other half female.

The renal patients were in a program of HD for 4 hours three times a week, continuous ambulatory peritoneal dialysis (CAPD) or automated peritoneal dialysis (DPA) and adherent to the treatment carrying out dialysis and its individual prescription. All the patients from the sample did not have any infectious or inflammatory process.

The project was approved by the Ethics Commitee in Research at the Santa Casa de Misericórdia of Pelotas Hospital and the Federal University of Rio Grande do Sul (UFRGS). The patients signed a post informed consent term in order to be interviewed and for the collection of the buccal mucosa cells. All of them answered the questionnaire, according to the protocol published by the International Commission for Environmental

Protection of Mutagens and Carcinogens (ICPEMEC, 1988). The information reliability was guaranteed.

The collection was carried out using the help of a wooden tongue depressor, previously moistened in still mineral water. The erosion in the oral cavity was made in the molar region in order to obtain abraded cells in the buccal mucosa. The first erosion was discarded. The wooden tongue depressor, with the scraping was put in a centrifuge tube with buffer phosphate, pH 6,8. Two tubes were used, one for the left side and the other for the right side of the oral mucosa. The material was then transported to the lab where it was processed.

Having being removed from the tubes, the wooden tongue depressors were centrifuged for 10 minutes at 1.000 rpm. The supernatants were retreated, leaving 0.5 ml of sediment and solution. 12 ml of fixative were added (methanol; acetic acid 3/1) and kept for 30 minutes in the freezer. It was then centrifuged again. The supernatant was discarded, leaving 0.5 ml of suspension. The procedure was repeated adding 8 ml of fixative and leaving at the end 0.3 ml of suspension. With the help of a Pasteur pipette, the solution was ressuspended and 3 drops were dripped in a pre heated lamina at 37°C. Afterwards, it was flambada and kept at room temperature for the whole night in order to dry. After the drying was done, the following sequence of hydrolysis was carried out: the lamina was put for 1 min in HCl (1N), drained and put in HCl (1N) at 63°C for 10 min, taken out and left to drain and cool down for 15 min. Once again, it was put in HCl (1N) for 5 min. It was washed 3 times with distilled water for 5 min each washing, kept at room temperature for 15 min and then put in Schiff dye for 2h30min in the dark. Having been removed from the dye, a solution of 80 ml of distilled water and 20 ml of buffer phosphate was added for 5 min (also in the dark). Afterwards, it was washed 3 times with distilled water, quickly and left to dry for a whole night. The following morning, the cytoplasm was dyed with fast-green.

The analysis of the cells was made using a common optic microscope, binocular, with objective of 100X and oculars of 10X. The macronucleus frequency and other nuclear anomalies (cells with broken eggs nucleus and binuclear cells), were registered in specific files. 2.000 cells per person were evaluated, with only the non-fragmented cells and non-crowding or overlapped being considered. The criteria used for the identification of a micronucleus were established by Picker and Fox (1986): (a) the micronucleus must have a regular contour, round or oval and must be inside the cytoplasm of a cell; (b) the

micronucleus must be Feulgen-positive and of an equal or lower intensity, the same texture and refraction of the principal nucleus; (c) the micronucleus must be smaller than the principal one, that is, its diameter must be 1/3 of the diameter of the principal nucleus; (d) be in the same focus plan; and (e) the micronucleus must be clearly separated from the principal nucleus. It was registered up to three micronuclei per cell, questionable micronuclei were not registered. For the statistics analysis a database was created using the statistics program SPSS, 10.0 "for Windows", the Student *t* test, two-tailed, the Mann-Whitney test and the Spearman correlation test, at a significant level of $p<0.05$.

Results

This study tried to evaluate the mutagenic potential determined by HD and DP treatment, to which the chronic renal patients are subjected. Table 1 shows the main characteristics which show the profile of the evaluated individuals and their controls that were matched for age and sex. The studied individuals were classified by type of treatment, sex, age, time of treatment, more frequent diseases, levels of creatine, salary group, use of smoking and alcoholic intake.

Table 2 shows the averages of the number of cells (in 2,000 cells analyzed) with micronucleus (MNC), binucleated (BNC), and broken egg nucleus (BEC) and total of micronuclei (TMN) of patients HD and DP, as well as the control groups, evaluated in this work. A significant difference ($p<0.01$) was found in patients HD (5.60±5,31 MNC; 5.60±5.30 TMN) and the control group (1.50±2.01 MNC; 1.76±2.24 TMN) in relation to the number of MNC and of TMN, but differences between the DP patients and its control group were not found.

Comparisons were made between the variables of sex, age (in the intervals: ≤50 e ≥51 years old), family wages (≤1 salary, from 2 to 4 salaries and ≥5 salaries), creatine level (≤10 and ≥10,01 mg/dl), use of smoking and alcoholic intake among the patients HD, DP and the control groups. The results found suggest that there is no association between these variables and the number of nuclear anomalies (Table 3 and Table 4).

The comparison between the variable time of treatment was also made, in the interval between ≤6 and ≥7 years old. The results have shown that there is an influence of the length of treatment and the number of alterations analyzed. The number of MNC (2.91±2.74) in the interval of ≤6 years old was significantly ($p<0.05$) lower than the value found

(8.89±5.96 MNC) in the interval of ≥7 years old of treatment. Also significant were the differences for the number TMN (≤6 years old: 3.09±3.08; ≥7 years old: 10.11±7.04; $p<0.05$) and for BEC (≤6 years old: 1.00±3.00; ≥7 years old: 3.78±5.09; $p<0.05$) for the patients in HD. The Spearman correlation coefficient (0.414) with $p=0.01$, has shown a positive correlation between length of treatment and the number of cells with MN. The same was not verified for the DP patients.

The possible influence of the level of creatine, use of smoking and alcoholic intake, age, and family wages were considered in the frequency of the nuclear alterations in relation to the sex in both types of treatment and in the controls. No relation was detected.

Discussion

According to Stopper *et al*. (2001), one of the consequences of chronic renal disease is the high risk of cancer. This happens in patients with IRC without dialysis treatment as well as in those who are kept in hemodialysis (Goodkin *et al.*, 2004). This fact can be related to failures in the repair mechanism and, in the latest studies, to the high frequency of MN found in these patients. The results of this study are in agreement with the findings of Stopper *et al*., mentioned above. On one occasion, a number of cells were found with MN significantly higher among the patients in HD than in the control individuals as well as the number of TMN.

In a previous investigation, Stopper *et al.* (1999) evaluated 19 patients in the final stages of renal insufficiency as well as 20 control individuals, observing a slight increase in the MN frequency, not significant, in these patients. Nevertheless, in this study the controls were not matched for age. In a second study, 16 patients with IRC in hemodialysis treatment were selected, 19 patients without dialysis treatment and 23 control individuals matched for age. In this evaluation the frequency of MN in BNC was analyzed obtained in the lymphocyte cultivation and a number significantly higher of MN among the patients kept in HD in comparison to the control group was observed.

In another study, Stopper *et al*. (2001) evaluated 26 chronic renal patients involved in treatment of HD, using the comet assay and also obtained significant differences between patients and controls.

Liu *et al*. (2001) carried out a study which evaluated the deletion of 4,977 bp of DNA mitochondrial of 162 patients with chronic renal disease, 125 patients being in HD and 37

in DP. They classified the studied individuals in age groups of 20-30, 31-40, 41-50, 51-60 and 61-70 years old and found in the chronic renal patients, at these age groups an incidence of deletion of 4,977 bp, respectively of 30.0%, 31.9%, 40.0%, 43.9% e 44.8%. In the controls the incidence was of 8.6%, 14.0%, 14.3%, 20.4% e 31.6%, respectively. The study showed, according to the authors, that there was a significant increase of deletions of 4,977 bp in DNA mitochondrial of the chronic renal patients in relation to the controls, due to the existence of factors that promoted genomic instability, leading to the occurrence of mutation.

Kan *et al.* (2002) using the comet assay, carried out a study about the DNA damage of patients kept in hemodialysis and found a significant increase in the breaks of DNA of chronic renal patients in hemodialysis. However, they did not find a positive correlation between the length of time (3.5 years) of hemodialysis treatment and the DNA damage that was found in the study of Stopper *et al*. (2001).

In this study, significant differences were found in the frequency of cells with MN, as well as in the number TMN in relation to the length of hemodialysis treatment. There was a significant increase ($p=0.038$) from 6 years of treatment onwards, confirmed by Spearman's coefficient of correlation ($p=0.01$). Kan *et al*. (2002) conclude that the length of treatment of the patients evaluated by them is very small, approximately 3.5 years, while Stopper *et al*. (2001) detected in their patients this increase from 10 years of treatment onwards.

This correlation between length of treatment and nuclear alterations can be explained by the fact that the blood of these patients, kept in hemodialysis treatment cycles, interacts with biocompatible membranes and in this process many reactive species of oxygen can be produced. It is also suggested that the dialysis process acts on the enzymes related to glutation, removing substrates and acting factors (Kan *et al.*, 2002).

Among the factors which were evaluated for their influence on the formation of MNC, BNC, BEC and TMN, one of the most relevant was the creatine level, as it is related to the severity level of chronic renal disease (Combe *et al*., 2004). The patients were classified in two levels of $\leq$10.00 mg/dl and $\geq$10.01 mg/dl. The results analyzed did not show differences in the number of nuclear anomalies. However, some studies report that the increase in the level of creatine is related to the number of MNC. Stopper *et al.* (2001) found a correlation between the level of creatine (severity of the disease) and the DNA damage, estimated using the comet assay. Nevertheless, Stopper *et al*. (1999) did not find

this correlation. In this second study, similar to this one, the level of creatine of the patients was very high, indicating the gravity of the disease in most of the patients.

Contrary to the results found in the patients in hemodialysis, significant differences were not found in DP patients. This fact can be due to the fact that all the patients were under treatment for a short time, at most for 6 years, which has limited this study. However, differences were not detected between the patients in HD and DP in relation to the cell alterations observed. It can be considered that the patients in DP were in a better general state of health than the ones in hemodialysis and that possibly the peritoneal dialysis plays a smaller genotoxic role.

Tarng *et al*. (2002) evaluated the oxidative DNA damage of leukocytes of peripheral blood, quantifying 8-hidroxi-2'-deoxiguanosina (8-HO-dG), using the electric chemical method in 24 control individuals, 22 patients with chronic renal disease without dialysis treatment and 42 patients in peritoneal dialysis. The highest average of 8-HO-dG was found in the DP patients, followed by the chronic renal patients and by the controls. The authors concluded that a significant increase in the level of 8-HO-dG occurs with chronic renal disease and that this level tends to increase the progression of the disease, meaning that the peritoneal dialysis treatment exacerbated this increase. Ishibashi *et al*. (2001) observed 8-HO-dG in the patients that were starting DP (3-5 months). However, 8-HO-dG was observed in higher numbers of mesothelial cells of patients of long term DP.

Concluding, the patients under hemodialysis treatment have shown more cells with micronuclei than their controls, indicating that there exist factors related to their condition that are increasing the nuclear anomalies, which are a consequence of alterations in the genetic material as well as failures in the repair mechanism. According to Stopper *et al.* (2004), the formation of reactive species to oxygen, due to chronic renal disease and the reactions of biocompatibility with the dialysis membranes, as well as a reduced antioxidant defense of these patients, can also contribute to the increase of chromosomal and/or genomic damage, taking into account that the formation of the micronucleus can result in breaks of the genetic material, as well as the loss of whole chromosomes which were not well distributed among the daughter cells during the mitosis process.

This fact was confirmed by the correlation between the length of treatment and the number of MNC, showing that the longer the treatment, the higher the genotoxic effect.

Factors, like levels of creatine, sex, age, family wages, use of smoking and alcohol do not influence the probable genotoxic action of the hemodialysis treatment.

The patients in DP did not present differences in the number of MNC in comparison with the control individuals, which suggest that this treatment is possibly less harmful for the patient. However, due to the short length of treatment of these patients, it is necessary to continue this study.

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