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GENOTOXIC EFFECT OF HIGH NITRATE WATER CONSUMPTION IN MEN AND WOMEN FROM NORTHERN MAR DEL PLATA, ARGENTINA

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ABSTRACT

Previous research has shown connections between the exposure to different agents and mutations in germinal and somatic cells. One of such agents, nitrate (NO_3), can be potentially genotoxic for individuals who drink high nitrate well water, and may cause a subsequent impact on their health and on the whole ecosystem. The aim of this research was to conduct genotoxicity assays in somatic cells from people living in the northern area of Mar del Plata city, Argentina. The purpose of these experiments was, in turn, to establish the possible relationships between mutagenic effects and high nitrate water consumption. To this end, different diagnostic tests were carried out to detect potential genetic and/or chromosomal alterations. A non-exposed population equal in age, gender and lifestyle formed the control group. Frequency of Sister Chromatid Exchange (SCE), Micronuclei (MN) and Chromosomic Aberrations (CAs) were determined. Furthermore, Cell Proliferation Kinectis was established through Replication Index (RI). Both a significant increase in MN frequency (Kruskal-Wallis H = 23.79, degree of freedom = 1, $p < 0.001$), and CAs presence (chromosome 9 mosaicism, ring chromosomes, fractures and chromosomal fragments) were observed in the exposed individuals compared with the control group. This genetic damage could be related to the exposure to high nitrate water, thus representing a potential risk to the health of the individuals concerned. However, it does not yet seem to be possible to conclude that this is the only reason that contributes to the mutagenic effects observed.

Key words: mutagenic agents, nitrate, chromosomal aberrations, genotoxicity, micronuclei.

RESUMEN

Se ha demostrado que hay relación entre la exposición a diferentes tipos de agentes y mutaciones en células somáticas o germinales. Uno de estos agentes, el nitrato (NO_3), puede ser potencialmente genotóxico en individuos que, por carecer de servicios de agua de red, consumen agua de pozo con alto contenido de este compuesto, lo cual, a su vez, puede tener impacto en su salud. El objetivo de este trabajo fue realizar ensayos de genotoxicidad en células somáticas de personas que viven en barrios de la zona norte de la ciudad de Mar del Plata, Argentina, con el fin de determinar si existe efecto mutagénico debido al consumo de agua con alto contenido de nitrato. Se realizaron pruebas diagnósticas para detectar posibles alteraciones genéticas y/o cromosómicas. Como control se utilizó una población no expuesta. Se determinó la frecuencia de Intercambio de Cromátides Hermanas (ICH), Micronúcleos (MN), el Índice de Replicación (IR) y la presencia de Aberraciones Cromosómicas (AC). Los resultados mostraron un incremento significativo de la frecuencia de MN (H de Kruskal-Wallis= 23.79, grados de libertad= 1, $p< 0.001$) y la presencia de AC (mosaicismo del cromosoma 9, anillos, fracturas y fragmentos cromosómicos) en las personas expuestas, lo cual evidencia daño del material genético. Este daño genético estaría relacionado con la exposición a aguas con alto contenido de nitratos, constituyendo un riesgo potencial para la salud de las personas expuestas. Sin embargo, no es posible aún inferir que esta sea la única causa que contribuye a los efectos mutagénicos observados.

Palabras clave: agentes mutagénicos, nitrato, aberraciones cromosómicas, genotoxicidad, micronúcleos.

INTRODUCTION

All along their life, organisms are exposed to physical, chemical or biological mutagens. In human beings, it has been observed that both the exposure to different kinds of agents and genetic alterations in germinal or somatic cells are related to congenital malformation development, sterility, autoimmune or degenerative diseases and cancer (Seoane and Dulout, 1999; Carballo *et al.*, 2001).

Nitrate intake above standard levels (45 mg/l or 45 ppm, according to Argentine Food Code, Fan *et al*., 1987) has been related to an increase observed in cases of gastric cancer (Bruning-Fann and Kaneene, 1993) and esophagus and stomach cancer (Anaya Pajuelo *et al*., 1999). The use of fertilizers mainly containing potassium and sodium nitrate produces groundwater aquifer pollution by nitrates. The use of this polluted groundwater as consumption water is therefore the principal means of nitrate exposure. On the other hand, and because nitrate is used as a preservative, it can enter the body through vegetable or meat product intake. Nitrate acts as a nitric oxide (NO) donor, a potentially genotoxic molecule. Chronic use of high nitrate concentrations in water may produce either cytogenetic effects, such as chromosome break (Tsezou *et al*., 1996) or micronucleated lymphocyte frequency increase (Andreassi *et al.*, 2001). Nitrates are quickly absorbed in the body at the gastrointestinal tract level. Microbial action produced both in the environment and in the human body reduces nitrates to nitrites (Eliano *et al*., 1995). Nitrites, in turn, combine with myoglobin to produce methemoglobin or secondary amides, the latter generating nitrosamines. Because methemoglobin cannot carry oxygen to tissues, blood nitrate excess may lead to methemoglobinemia with serious toxic effects or even death in children (Walton, 1951; Sadeq *et al*., 2008). Nitrosamines can methylate DNA and alter its sequence, thus increasing abnormal or neoplasic cell production. Carcinogenic mechanism is more related to the efficacy with which the excision mechanism (elimination) of the nitrogenous bases alkylated by N-nitrous compound works than to the alkylation level itself in a specific location (Loera Gallardo, 1985). Nitrate carcinogenic and teratogenic effect has been observed in animals (Tapia, 2000; Manassaram *et al.*, 2005) whereas in human beings the magnitude of its potential risk has not been fully elucidated to date.

In view of the above, it can be concluded that in the last decades human health has been negatively affected by agro-industrial activity, which is responsible for the exposure to chemical products and genotoxic agents. It thus becomes necessary to carry out genotoxicity assays on a regular basis in order to determine genetic damage level in a given population. In line with this, individuals in risk of suffering alterations that may modify their genetic stability should be monitored (Zalacain *et al*., 2005). The purpose of the present study was therefore to carry out a minimal test set of genotoxicity assays including Sister Chromatid Exchange (SCE), Micronuclei (MN), Replication Index (RI) and presence of Chromosomal Aberrations (CAs) to somatic cells from individuals living in the northern area of Mar del Plata city, Argentina, in order to determine the potential mutagenic effect derived from consumption of high nitrate content water.

MATERIALS AND METHODS

This research was carried out in Alto Camet, Las Dalias and Parque Peña, three neighborhoods located in northern Mar del Plata city, Buenos Aires province, Argentina, which cover a surface of approximately 750 hectares, and with a total population of approximately 15,000 people who consume high nitrate water. Therefore, samples containing up to 137 ppm NO_3 were collected from this water. These samples considerably exceed tolerable standards (45 ppm) (Lasta *et al.*, 2003; Manrique, 2007).

Before sample collection, participants were informed on the steps to follow in the present study as determined by the informed consent approved by the Bioethics Committee of the *Asociación de Genética Humana* (AGHU) in Mar del Plata, and were invited to participate in the study. Samples of peripheral blood from 19 randomly chosen individuals were taken: 7 males and 12 females with an average age of 52.8 ± 12.04 (exposed group). The high nitrate water exposed group was formed of these individuals who proved to keep a record of at least one-year-long residence in the above mentioned neighborhoods. The control group, in turn, was formed of 19 individuals (7 males and 12 females with an average age of 50.4 ± 11.66) who

used water with the allowed level of nitrates. Both groups were gender- and age-matched.

Frequency of SCE, MN and CAs in the exposed and control groups was analyzed in order to study the potential genotoxic damage induced by exposure to polluted water. Peripheral blood samples (4 ml.) were taken using disposable syringes with norheparin (100 U). Fluorescence-plus-Giemsa technique (FPG) was applied for SCE (Perry and Wolf, 1975). This technique consists in differential staining, which, after three *in vitro* cell division cycles, reveals break (damage) and recombination (repair) points. Cells were cultured in a complete medium (RPMI medium 1640 (GIBCO) with 10 mg/ml phytohemagglutinin (PAA) and fetal bovine serum (PAA), supplemented with a 10 mg/ml final concentration bromodeoxyuridine (BrdU) (SIGMA) during 72 hours at 37º C. Cells were then harvested through the addition of colchicines (SIGMA), subsequently incubated in 0.075M KCl hypotonic solution and fixed in Carnoy solution (methanol/ acetic acid 3:1). Chromatid differential staining was performed with Hoechst 33258 (SIGMA) coloring and 2% Giemsa (BIOPUR) staining solution in sheets exposed to UV light for 4 hours. Frequency of SCE and CAs was determined and CPK (cell proliferation kinetics) was established through RI.

A 50 cell-count was performed per individual in the metaphase stage of second mitotic division so as to determine the number of SCE. To study CAs, 100 cells in the metaphase stage of the first mitotic division were analyzed per culture and individual. The amount of AC was estimated for each cell analyzed (Gadano *et al*., 1998). Both the extra chromosomes and the chromosomal fragments found were identified via G-band technique (Seabright, 1971; Barch *et al.* 1997). In order to estimate RI, 100 consecutive cells in the metaphase stage were analyzed per culture and individual. In addition, the proportion corresponding to cells in first (M_1), second (M_2) or third (M_3) mitotic division was counted, RI being determined as RI = ($1M_1$ + $2M_2$ + $3M_3$)/100.

Micronuclei were studied without the addition of cytochalasin B whose reagent was originally used for isolated lymphocytes, and the mayority of laboratories subsequently used it for whole blood cultures (Fenech *et al*., 1999). This is so because cytochalasin improves the sensitivity method by blocking cytokinesis. However, the extent to which mutagen (cytochalasin B) exposure leads to MN formation already *in vivo* or to MN formation *ex vivo* during cell culture as a consequence of persisting DNA damage remains unknown (Speit *et al*., 2011). Cytochalasin B application also has the following drawbacks: 1) it may interfere after MN induction by chemical tests, i.e., it behaves similarly to spindle poison; 2) it may interfere with other cytokinesis inhibitors; and 3) cytochalasin B cytotoxicity varies among cell lines and, sometimes, even among subtypes of the same cell line (Fenech and Morley, 1985a,b; Fenech, 1993; Kirsch-Volders *et al*., 2000). Furthermore, MN already induced *in vivo* can be determined by scoring MN in mononuclear lymphocytes 24 hours after lymphocyte culture initiation (i.e. in lymphocytes not yet divided) (Speit *et al*., 2011). In the present study, the following criteria for MN identification were followed: 1) MN were not refractory; 2) their color was the same or lighter than that of the nucleus from which they had originated; 3) their diameter ranged from 1/16 to 1/3 of the average diameter of the cell nucleus from which they could have originated; 4) they did not overlap with their main nucleus; and 5) they were located at a maximum distance of 3 to 4 nuclear diameters from the cell from which they had originated. Blood samples were cultured in a complete medium, as the one specified above, during 24 h at 37º C in the presence of 5% CO_2. Samples were harvested in the absence of colchicine, incubated in hypotonic solution and subsequently fixed in solution. Staining was made with 3% Giemsa in 0.6M to pH 7 Sorensen´s buffer. In addition, MN percentage in 1,000 mononucleated cells analyzed per individual was determined. Non-parametrical Kruskal-Wallis test was applied for the statistical comparison of the two groups (control vs. exposed) for SCE, RI and MN. The test was performed with SPSS software (10.0 version).

RESULTS AND DISCUSSION

Exposure to high nitrate water in northern Mar del Plata showed evidence of genetic damage in the group exposed to this chemical compound, with a statistically significant increase in MN and CAs

(cell mosaicism, chromosomal fragments and ring chromosomes). The present study revealed that all control individuals yielded standard MN values (from 1 to 3 MN/1,000 mononuclear cells, according to the Human Micronucleus Project, Fenech *et al.*, 1999), whereas 17 out of the 19 exposed individuals showed values above the normal range. The mean number of MN in the exposed individuals (6.84 ± 1.92) increased significantly with respect to that of the control group (2.58 ± 1.17) (Kruskal-Wallis H = 23.79, degree of freedom = 1, p = 0.001) (Fig. 1). The analysis of MN by gender revealed a statistically significant increase in the exposed individuals of both genders, females accounting for 66.26% of such increment (Kruskal-Wallis H = 17.4175, degree of freedom = 1; p=0.0000) and males accounting for 54.20% (Kruskal-Wallis H = 5.2662, degree of freedom = 1; p = 0.0217). These values should be taken into account because they are associated to chromosomal loss or breakup, both having an impact on human health and consequences on the individual and his descendants (teratogenesis to perinatal or infant death, or even neoplasias) (Mudry and Carballo, 2006). In this respect, the use of Fluorescent *in situ* Hybridization (FISH) could be useful to differentiate if the MN defined by the monitored genotoxic action corresponds to a chromosome fragment (clastogenic effect) or to a whole chromosome (aneugenic effect).

Chromosomal aberrations were detected in individuals from the exposed group. In three cases, CAs corresponded to mosaicism of chromosome 9. One individual showed 47,XX,+9p(2)/47,XX,+9(4)/46,XX(94) karyotype. The other two exhibited 47,XX,+9(2)/46,XX(98) karyotype and 47,XX,+9(1)/46,XX(99), respectively. The presence of an extra chromosome 9 was determined by G banding technique. Likewise, a fourth individual revealed chromosome rings of different sizes (3 large and 1 small) 47,XY,+r(?)(4)/46,XY(96). The chromosomes to which these rings corresponded could not be determined. In other cases as well as in the individuals exposed, chromosome fractures and fragments were observed. However, no dicentric chromosomes, chromatid breaks or multirradial chromosomes were observed in the exposed individuals analyzed (Table 1). It could thus be inferred that there is a likely relationship between nitrate-contaminated water intake and the above-mentioned anomalies. In addition, such relationship could be related to a possible mutagen activity of nitrites/nitrates. The frequency of chromosome 9 trisomy in pure line (all cells) is very low in human beings who have a low survival rate at birth as a result of the multiple malformations and lethal effects caused by this genetic disorder (Inostroza *et al*., 2002; Rodríguez, 2005). Individuals with chromosome 9 mosaicism in the exposed group had no malformations. The low mosaicism of chromosome 9 observed in the present study could be acquired by the exposure to mutagen agents.

Replication Index decreased in the exposed individuals (1.19 ± 0.3) with respect to the control group (1.27 ± 0.22). However, these differences were not statistically significant (Kruskal-Wallis H = 3.2465, degree of freedom = 1; p = 0.0716) (Fig. 2). The major difference in RI was observed in males (Kruskal-Wallis H = 0.268, degree of freedom = 1; p = 0.605) with respect to females who presented no statistically significant differences (Kruskal-Wallis H = 2.204, degree of freedom = 1; p = 0.138).

Frequency of SCE in the exposed individuals group (4.48 ± 2.98) was higher than that in the individuals belonging to the control group (3.71 ± 1.62), however, SCE frequency showed no significant statistical differences (Kruskal-Wallis H = 3.1921, degree of freedom = 1; p = 0.074) (Fig. 3). The analysis of SCE frequency for both genders revealed that the exposed females outnumbered the non-exposed ones by 30%. As far as males are concerned, the increase was just 7%. Still, the statistical analysis showed no significant differences between SCE frequencies based on gender neither for females (Kruskal-Wallis H = 2.1182, degree of freedom = 1; p = 0.1456) nor for males (Kruskal-Wallis H = 1.0714, degree of freedom = 1; p=0.3006) for this result.

In the present study, the number of SCE by metaphase ranged from 3 to 5 in the control group, and from 5 to 8 in the exposed group. Even though the values are within the basal range and are not statistically different, there is a trend towards a SCE frequency increase in the individuals of the exposed group. Previous research has reported a close relationship between SCE high frequencies and cancer predisposition (Spitz and Bondy, 1993; Cortés Gutiérrez *et al*., 2000). Thus, although this increase is not statistically significant in the SCE

frequencies of exposed individuals, it should not be disregarded and extensive research efforts should be devoted in the near future including a larger number of participants and other variables so as to rule out or identify a possible correlation between high nitrate water exposure and carcinogenesis.

Several factors could affect the parameters analyzed and should be taken into account. It was shown that MN frequency correlated with age in both genders. It was also observed to be affected by dietary factors (Fenech *et al.,* 1999) and occupational exposure (Kirsch-Volders *et al.*, 2000). Further studies have suggested that the average frequency of micronucleus cells does not differ between smokers and non-smokers and between males and females (Sarto *et al.,* 1990). In other words, findings are, in general, contradictory and therefore further studies should be carried out. Moreover, in spite of the fact that age does not play a significant role in cytogenetic manifestations (Pérez-Herrera *et al.*, 1999; Ceballos-Quintal *et al.*, 2002) and although there seem to be no relevant gender-related differences in SCE (Pérez-Herrera *et al*., 1999), an early study contradicted these findings (Verma and Babu, 1995). Other variables related to lifestyle which could be considered genotoxic are tobacco use and alcohol ingestion as they are known to affect chromosomal stability. Alcohol seems not to induce SCE formation although it could be responsible for structural CAs (López *et al*., 2001). In the present study, although individuals from both groups were asked about tobacco use, no obvious conclusions could be drawn.

Results from the present study are consistent with those of previous research reporting that high nitrate levels in water could induce genotoxic effects (Tsezou *et al.,* 1996) and cytotoxicity as well as a slowing down of the cell cycle (Andreassi *et al.*, 2001). A pattern of genetic damage (MN increase and trisomies) similar to the one observed by high nitrate water use was found in a northern Argentine population exposed to arsenic-contaminated water (Dulout *et al*., 1996). On account of this, both compounds are likely to cause similar deleterious effects on human health.

Taken together, results from the present study lead us to infer a relationship between high nitrate water use and genetic damage, the former being therefore a potential threat to the health of those living in Mar del Plata and drinking nitrate water as a part of their daily diet. Still, it cannot yet be concluded that high nitrate concentration in water is the only cause of the mutagenic effects observed although this research tried to establish nitrate concentrations as the only difference between the exposed and control groups.

Furthermore, taking into account the direct action human beings exert on the environment (use of fertilizers and compost, among others), their lifestyles (cured meat consumption and nitrates use as food additive), and the high nitrate levels in water, soil and food, they all contribute to creating a continuous source of undesired contamination. As a result of the increasing number of human beings exposed to nitrate/nitrite polluted water, it becomes necessary to carry out more thorough studies at local level. Such studies will greatly contribute to either establishing or discarding causal relationships among these substances and their effects at a molecular and cellular level, and to determining their impact on individual and population health within Argentine communities. Thanks to the results from the present study, regular tap water supply was secured to one of the affected communities analyzed.

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Individual	Numerical CAs	Chromosome Break	Chromosomal Fragment	Chromosome Rings	Gaps
1	47,XX,+9p(2)/47,XX,+9(4)	-	-	-	-
2	47,XX,+9(1)	-	1 corresponding to chromosome 13	-	-
3	47,XX,+9(2)	1	-	-	-
4	-	-	-	4 (3 small and 1 large)*	-
5	-	-	-	-	2
6	-	-	-	-	3
7	-	-	-	-	1
8	-	-	2	-	-

Table 1. Types and amounts of CAs in individuals exposed to high nitrate water

Number of cells analyzed per individual = 100. No dicentric chromosomes, chromatid breaks or multirradial chromosomes were observed in the exposed individuals analyzed. Individuals 9 to 19 were not included in the table because they evidenced no chromosomal abnormalities.

*The chromosomes to which these rings corresponded could not be determined.

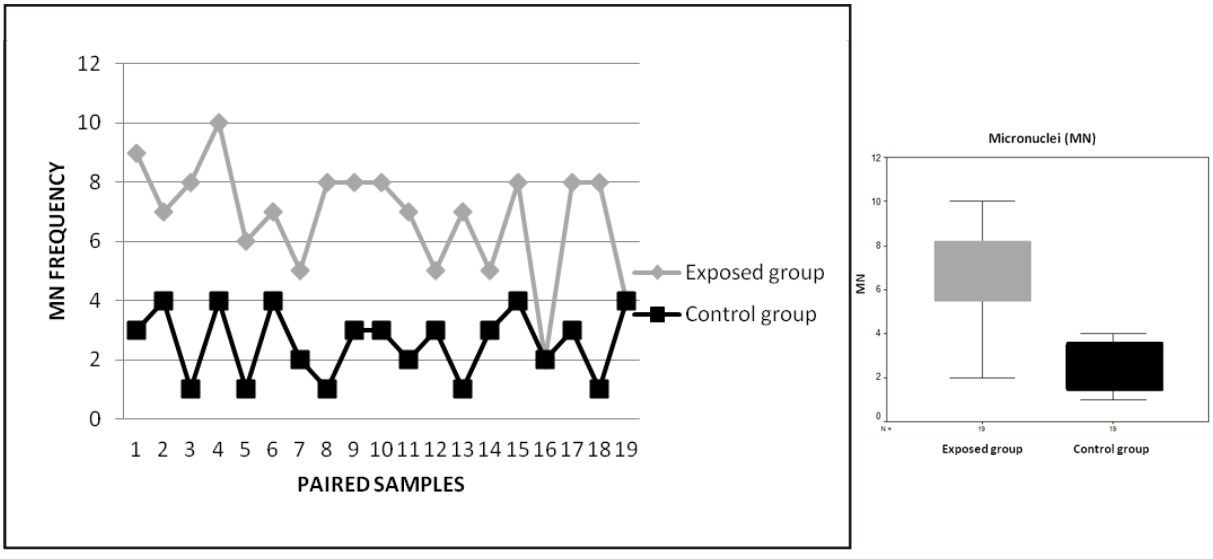

Figure 1. Frequency of MN in males and females: control and exposed groups in relation to high nitrate water consumption in the northern area of Mar del Plata city.

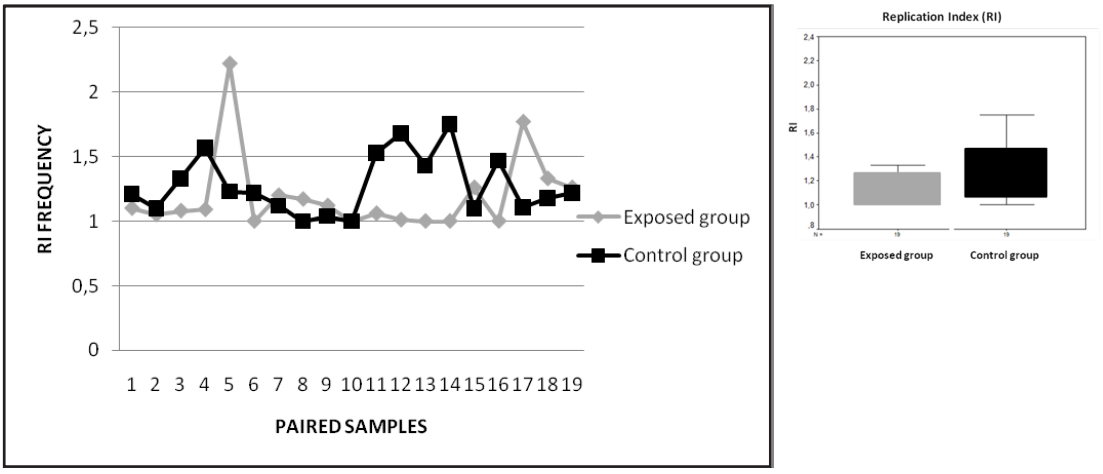

Figure 2. Frequency of RI in males and females: control and exposed groups in relation to high nitrate water consumption in the northern area of Mar del Plata city.

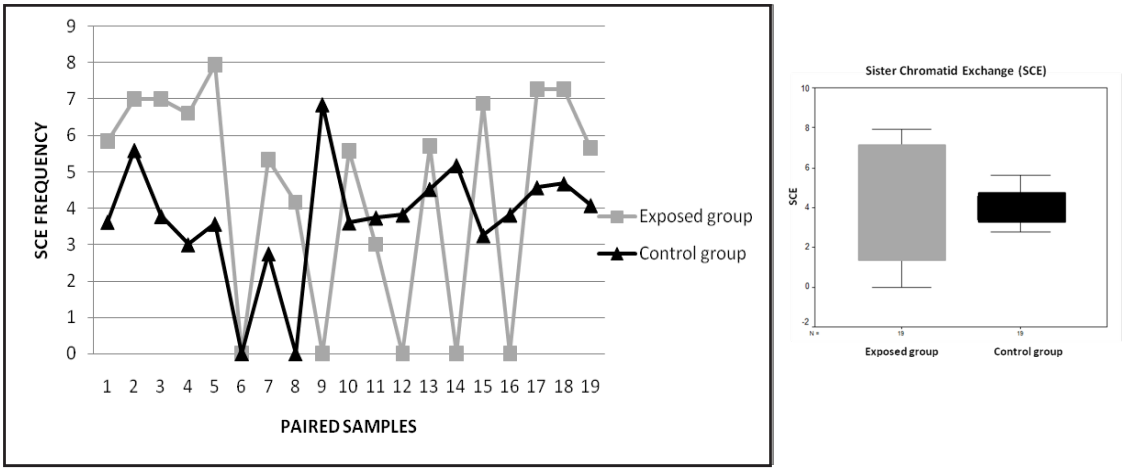

Figure 3. Frequency of SCE in males and females: control and exposed groups in relation to high nitrate water consumption in the northern area of Mar del Plata city.

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