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Full Length Research Paper

Eye drops prescription pattern in patients with glaucoma

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Glaucoma is a major public health problem, being the leading cause of irreversible visual impairment worldwide. In most cases, glaucoma is associated with intraocular pressure (IOP) levels that are above those considered normal (IOP ≥ 21 mmHg). The aim of the present work is to describe the pattern of eye drops prescription in patients with glaucoma in the state of Sergipe, Brazil. A descriptive cross-sectional study with retrospective documentary analysis, developed at the Eye Institute of Sergipe was performed. Of all glaucoma patients, 41.5% had a family history of the disease. Among the popular drugs, Latanoprost 0.005% is the most requested totaling 34% of prescriptions. In the Region 2, a significant association (χ^2= 48, 45; p<0.05) was observed between monotherapy and high IOP. The present study showed that the pattern of eye drops prescription was as recommended by the Brazilian Society of Glaucoma. Although, these medications were distributed freely, most patients with glaucoma have high IOP. Therefore, considering the importance of glaucoma as the second cause of blindness worldwide it is essential to carry out studies that evaluate the factors that influenced the effectiveness of eye drops use for treatment of glaucoma.

Key words: Glaucoma, ophthalmic solutions, prescription drugs.

INTRODUCTION

Glaucoma is a silent chronic disease that is characterized by progressive alterations of the optic disc, retinal nerve fiber layer, irreversible loss of vision and damage to the optic nerve. In most cases, glaucoma is associated with intraocular pressure (IOP) levels that are above those that are considered normal (IOP ≥ 21 mmHg). The total number of patients (aged 40 - 80 years) with glaucoma worldwide was estimated to be 64.3 million in 2013 increasing to 111.8 million in 2040, especially in Asia, Africa and America (Tham et al., 2014). According to the World Health Organization (WHO), glaucoma accounts for 13% of global blindness, and 2.4 million new cases occur every year (WHO, 2004; WHO, 2013).

Although, glaucomatous damage is deemed irreversible, approximately 90% of the blindness caused by glaucoma could be avoided with early diagnosis and treatment (Susanna, 2015, UN, 2009). Thus, glaucoma is a serious public health problem (William et al., 2014). Depending on the condition, medical, laser, surgical treatments, and antiglaucoma therapy can be used.

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A great number of topical medications are available for use in glaucoma treatment. These eye drops cause IOP reduction by either decreasing production of aqueous humor from the ciliary body, or increasing outflow of the aqueous humor through the trabecular meshwork or uveoscleral pathway, the natural drain of the eye (Sun et al., 2015).

In developing countries, including Asia, Africa and South America, most glaucoma patients are unconscious that they have the disease and visual injury that is also prevalent and severe (Thomas et al., 2012; Leite et al., 2011). Moreover, two reviews highlight that lack of adherence to the antiglaucoma therapy may culminate in the patient's vision loss (Castro, 2009; Leite et al., 2011). The low adherence can be explained because the glaucoma medications do not improve vision, may have important side effects and are relatively expensive (Leite et al., 2011).

Data on the prevalence of the disease in Brazil are scarce (Leite et al., 2011; Castagno et al., 2009; Tham et al., 2014). Sakata et al. (2007) reported that the prevalence of glaucoma in South Brazilian population among people over 40 years old was 3.4%. According to Stillitano et al. (2005), the antiglaucoma therapy in Brazil is expensive, and only a fraction of the economically active population is treated. As reported by Brazilian Ministry of Health and Brazilian Council of Ophthalmology, about 2% of Brazilian population has glaucoma. Although since recently the Brazilian population has received free eye drops, more than one fifth of the glaucoma patients do not follow the drug treatment in an adequate technique (Castro, 2008, 2009). Then, this study aimed to characterize the use of eye drops in patients with glaucoma in part of Brazilian Northeast.

MATERIALS AND METHODS

Study design

The present study is a descriptive research that was based on secondary data extracted from medical records that were used by an ophthalmic reference center for glaucoma patients from the Brazilian Public Health Care System (SUS) in the State of Sergipe. The center attends to about 10,000 elderly patients, and its health care team consists of four physicians, one nurse, one nursing technician, and one pharmacist. Sergipe is the smallest state in Brazil, located in the Northeast Region, this is the second most populous and poorer region of the country.

Characterization of Brazilian health care

Since 1989, all 200,000 Brazilian have been entitled to free health care at primary, secondary, and tertiary level through a national health system that is unique in Latin America, being funded by taxes and social contributions, such as social security payments. The Brazilian Public Health Care System (SUS) has decentralized care coverage based on Family Health Strategy that proved to reduce common diseases, mortality, and reductions in unnecessary admissions (Victoria et al., 2011). This Health Care System is divided in territories called Health Regions.

The Health Regions represent the integration of actions and health services of the federative institutions in a territory to qualify the SUS management, and also to ensure integrity. It must be able to cover and solve almost all health needs shown by the population of the region. In this study, the Health Regions were divided into three regions. Each region has a specific number of municipalities: Region 1 (15), Region 2 (11) and Region 3 (10). The division of regions considers geographical proximity defined by cultural, economic and social aspects, provision of health services, communication networks and infrastructure (Duarte et al., 2015; Santos, 2015). In this context, the Brazilian Public Health Care System (SUS) has a Glaucoma Patient support program present in all States and municipalities in Brazil. This program aims to manage patient support, increase public access, and generate mechanisms that can expand the diagnosis, monitoring, and treatment of these patients (Brazil, 2002a,b).

Databases and analysis instrument

For monitoring glaucoma patients, databases including information on diagnosis and treatment were created. The databases included users who were diagnosed with primary open-angle glaucoma and who were treated between June 2011 and March 2012 in 36 municipalities. These municipalities covered 48%, nearly 1,000,000 inhabitants of the State of Sergipe. The analysis instrument was structured by researchers to evaluate the study population's profile. The database analysis was performed by a team consisting of a pharmacist (C.A.S.S.) and a Pharmacy undergraduate student (C.B.R.) of the Federal University of Sergipe. Both researchers were trained for six hours in order to be able to evaluate the databases. The data collected included a sociodemographic profile for each patient (name, age, gender, and length of stay in the program), the drugs prescribed for ophthalmic use, comorbidity, prescription patterns and outcomes of the users that were monitored for ten months in an ophthalmic reference center. At the end of the study, all of the instruments used were reviewed by the two researchers (F.A.S. and D.P.L.J), the results were further analyzed.

Population of study

Data collection was obtained from medical records of 9,308 patients who were diagnosed with glaucoma. In order to characterize the prescription patterns of eye drops, the prescribed drugs and types of therapy were analyzed. In Brazil, the respondents reported their ethnicity for classification according to the Brazilian Institute of Geography (IBGE).

Statistics analysis

The data was analyzed with BioEstat 5.0 and Epi info for Windows, IOP (CDC, 2012) prevalence estimates were examined according to age and monotherapy (the use of a single drug to treat a particular disorder or disease). In order to evaluate the statistical significance of the association between these variables and IOP, Mantel-Haenszel chi-square test was employed. A confidence interval of 95% was set for all of the statistical tests performed, and the differences were considered statistically significant if the p value was equal to or lower than 0.05.

Ethical aspects

The study was approved by the research ethics commission of the

Table 1. Frequency distribution of the variables gender, ethnic group, number of medicines used, existence of comorbidities, familiar glaucoma in the studied population. Sergipe June 2011 to March 2012.

Variable	Frequency	%
Gender		
Male	3222	35
Female	6062	65
Ethnic group		
Brown	4607	49.5
Black	2860	30.7
White	1831	19.6
Indian	5	0.05
Yellow	3	0.03
Number of medicines		
1	8536	92
2	686	7
3	87	1
Co-morbidades		
Hypertension	2454	49
Migraine	1450	29
Diabetes	932	18
Asthma	184	4
Familiar Glaucoma		
No	5442	58.3
Yes	3867	41.5

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RESULTS

In this study, 65% of the patients were female. Most of the population reported themselves as brown-skinned (49%), 31% as black, and 20% were self-declared white as shown in Table 1. The average age of the population from the three regions was 58 years old (standard deviation: 15.81). Among the comorbidity more commonly reported by patients from the three regions, arterial hypertension, migraine, and diabetes had higher percentages of occurrence (49, 29, and 4%, respectively). In addition, 41.5% of the patients reported a family history of glaucoma as shown in Table 1.

When comparing the average number of prescribed eye drops used by patients in the three regions, the majority (93% of the prescriptions) were for only one drug as shown in Figure 1. Thirteen drugs were used for the treatment of glaucoma as shown in Table 2, and prostaglandin analogs (Xalatan®) accounted 34% of the total as shown in Figure 2. In Region 2, a statistically significant association (c^2 = 48.45; $p < 0.05$) was observed between monotherapy and abnormal IOP as shown in Table 3. Therefore, individuals with glaucoma who were treated with monotherapy were 1.76 times more likely to present an IOP ≥ 21 mmHg compared to individuals who were treated with more than one drug as shown in Table 3.

DISCUSSION

In this study, majority of the population was composed of female patients. Though, no associations between gender and the percentage of individuals affected by glaucoma have been reported, and the prevalence of the disease is similar in both genders (Lima et al., 2010). This phenomenon may be related to observations that women use healthcare services more often, especially for social and reproductive issues, which results in a greater demand of drugs by this gender (Quigley, 2006; Le A et al., 2003; Travassos et al., 2002; Fleith et al., 2008).

Regarding ethnicity, the majority of patients with glaucoma who were included in this study reported themselves as black or brown-skinned. According to the Brazilian glaucoma society (Brazil, 2009; Brazil, 2011; BCO, 2015), morbidity develops earlier in black-skinned individuals compared to whites. In this study, the majority of the patients were brown-skinned, and this could be related to the racial miscegenation in the state of Sergipe.

The most frequent comorbidity was arterial hypertension, but no correlation was found between glaucoma and systemic arterial hypertension. Although this study did not find any associations between these variables, several studies have reported a correlation of increased blood pressure and glaucoma susceptibility (Bonomi et al., 2000; Dielemans et al., 1995; Goldberg et al., 1981; Sun et al., 2012). The blue mountains eye study and rotterdam eye study reported an association between systemic arterial hypertension and glaucoma susceptibility (Mitchell et al., 2004; Hulsman et al., 2007). Although the percentage of patients with diabetes and glaucoma was small, the role of diabetes is important in glaucoma. Previous studies (Kanamori, 2004; Pasquale, 2006; Minwen, 2014) have suggested that people with diabetes are more likely to have high IOP and glaucoma. In a cross-sectional study, Bonovas et al. (2004) suggested that increased IOP is linearly correlated with the severity of diabetes.

This study showed that physicians initially prioritized treatment with only one drug, typically prostaglandin analogs, because prostaglandins and beta-blockers are recommended in the initial treatment of glaucoma (Heijl, 2002; Van der Valk et al., 2005; Li et al. 2006 Brazil, 2009; Burr et al., 2012; Estacia and Tognon, 2008). However, the analysis of the three regions showed that most of the patients in region 2 who were treated with only one drug had higher IOP values than the

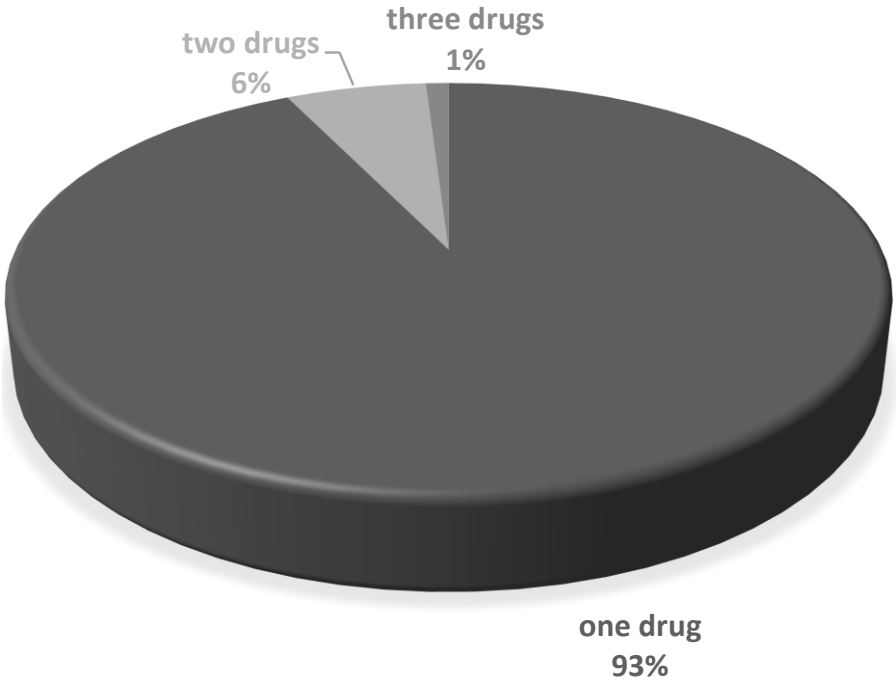

Figure 1. Percentage of drugs used in the treatment of glaucoma. Aracaju, June 2011 to March 2012.

Table 2. Prescribed drugs for glaucoma's treatment.

Marketed drug	Active substance	Drug group	Frequency	Percentage
Azopt®	Brinzolamide 1%	Carbonic anhydrase inhibitor	62	1
Combigan®	Brimonidine tartrate 0.2% + Timolol maleate 0.5%	α_2-Adrenergic agonist and β-Adrenergic blocker	235	3
Drusolol®	Dorzolamide hydrochloride 2% + Timolol maleate 0.5%	Carbonic anhydrase inhibitor and β-Adrenergic blocker	46	1
Ganfort®	Bimatoprost 0.03% + Timolol maleate 0.5%	β-Adrenergic blocker and Prostaglandin analogue	67	1
Timolol maleate	Timolol maleate 0.25%/0.5%	β-Adrenergic blocker	2417	28
Brimonidine tartrate	Brimonidine tartrate 0.2%	α_2- Adrenergic agonist	744	9
Xalacom®	Latanoprost 0.005% + Timolol maleate 0.5%	Prostaglandin analogue and β-Adrenergic blocker	927	11
Xalatan®	Latanoprost 0.005%	Prostaglandin analogue	2944	34
Cosopt®	Dorzolamide hydrochloride 2% + Timolol maleate 0.5%	Carbonic anhydrase inhibitor and β-Adrenergic blocker	15	1
Duo-travatan®	Travoprost 0.004%+ Timolol maleate 0.5%	Prostaglandin analogue and β-Adrenergic blocker	362	4
Lumigan®	Bimatoprost 0.03%	Prostaglandin analogue	186	2
Ocupress®	Dorzolamide hydrochloride 2%	Carbonic anhydrase inhibitor	65	1
Travatan®	Travoprost 0.004%	Prostaglandin analogue	470	4

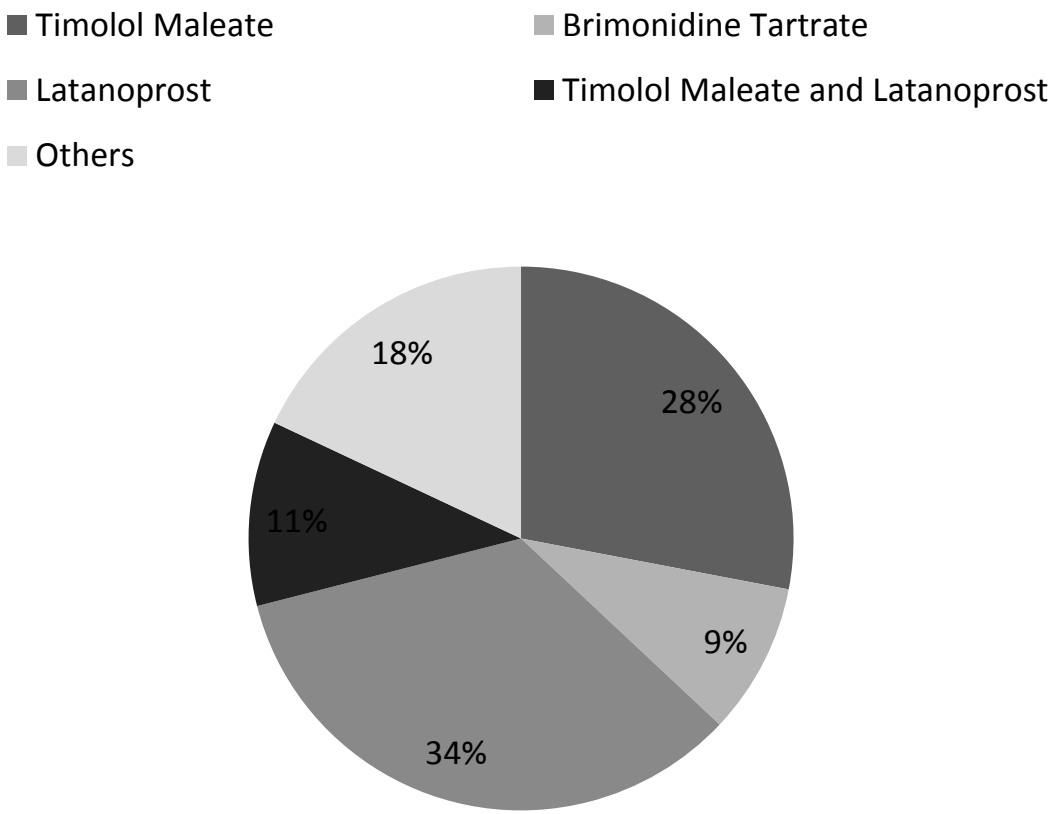

Figure 2. Percentage of most prescribed drugs. Aracaju, June 2011 to March 2012.

Table 3. Distribution of final IOP, according to the variable monotherapy. Estância, June to March 2012.

Monotherapy	IOP (mmHg)		Prevalence (%)	RP[a]	χ^{2b}	p-value
	≥21	< 21				
Yes	483	988	32.8	1.76	48.45	0.0000001
No	116	84	58	1.00		

recommended standards. A reason for the lack of IOP control in this study may be a lack of adherence to the pharmacotherapy, as most of the patients reported eye irritation from the eye drops during the beginning of treatment in the medical records. In addition, several studies have reported that some patients who are diagnosed with glaucoma do not respond to first-line treatment drugs, and two or more antihypertensive drugs are required to achieve the target IOP (Cheng et al., 2012; Broadway and Cate, 2015; Kass, et al., 2002).

When analyzing the final IOP of the patients from Region 3, age was observed to be a risk factor for increased IOP. This conclusion was found to be in agreement with other studies that reported a positive association between high IOP and age (Kawai, 2001; Quigley, 2006; Schneck et al., 2004). Studies that were performed in Australia and the US have shown that the prevalence of the disease steadily increases with each decade of life, starting at 55 years old (Mitchell et al., 1996; Wensor, 1998; Quigley et al., 2001). Owsley (2011), in his research, describes the problems caused in vision due to aging and its impact on economic and personal life of the population.

The present study showed that, even though eye drops are prescribed for the treatment of glaucoma as recommended in the literature, patients have high IOP in the analyzed cities. These results could be associated to low adherence to glaucoma pharmacotherapy. Several studies have demonstrated that roughly 50% of patients were not adherent to their medication over 75% of the time (WHO, 2000; Okeke et al., 2009; Robin and Grover, 2011; Costa et al., 2011). Cohen et al. (2014) related that low adherence to glaucoma pharmacotherapy is related to patients, medication, physician and environmental

factors. According to Robin and Grover (2011), some barriers influenced to adherence, such as forgetfulness, other priorities, lack of information and emotional factors. Another study carried out by Newman-Casey and colleagues (2015) also pointed to the difficulty with drop administration and the medication schedule as barriers associated with poor adherence, even in developing countries.

This study showed the prevalence among brown-skinned and elderly patients that use monotherapy of eye drop glaucoma. Although, these medications were distributed freely, most patients have high IOP. Because of the prevalence of glaucoma and the high costs of this disease, it is essential to perform further studies that adequately evaluate the factors that influenced the effectiveness of the use of eye drops, especially to patients' knowledge and attitudes, in the treatment of glaucoma.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Preclinical evaluation of repeated dose toxicity and anxiolytic activity of monoterpene R - (+) – limonene via inhalation

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R - (+) - limonene is a monoterpene found in several plant species. Studies have shown that plants that have this compound has anxiolytic activity. This study aimed to investigate the anxiolytic and toxicological activity in rats after inhalation of R - (+) - limonene for 30 min. For the anxiety tests using Elevated Plus Maze (EPM), concentrations of 1.0, 2.5 and 5.0% were determined. From the results, it was observed that the last concentration had achieved anxiolytic effect. Based on these results, it was found by biochemical and hematological tests, toxicity of 5% concentration in the period of 30 days with minor significant changes. In the hematological parameters, MCV, MCH and MCHC values were significant when compared to the control group, but were within the normal range when compared to values found in literature. In the biochemical parameters, changes occurred in AST and LDH levels. Elevated LDH levels can cause destruction of erythrocyte precursor cells in the bone marrow. In turn, AST may be associated to small changes in the liver. Therefore, the results demonstrated an anxiolytic activity with low toxicity at dose of 5% R - (+) - limonene via inhalation for 30 min.

Key words: R-(+)-limonene, inhalation, biochemical, hematological, anxiolytic, toxicity.

INTRODUCTION

The therapeutic effects of essential oils (EOs) on physical and psychological disorders are well known. Studies with aromatherapy have reported medicinal properties of aromas that have been used for thousands of years by humans in the treatment of various diseases (Kutlu et al., 2008). EOs, administered via inhalation, stimulate the olfactory nerves that have an effect on the brain, which results in behavioral changes in animals and humans (Su et al., 2009; Lawless, 2002). Their use may represent an alternative method for the treatment of disorders such as

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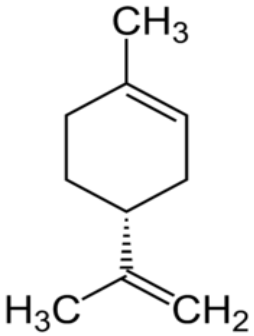

Figure 1. Chemical structure R-(+)-limonene.

anxiety (Almeida et al. 2004) and seizures (De Sousa et al., 2006).

Monoterpene R - (+) - limonene (1-Methyl-4- (1-methylethenyl) -cyclohexene) (Figure 1) is present at high proportions in citrus fruits and is marketed by many EO manufacturers (Malhotra et al., 2009). EOs are found in various plants such as *Lippia alba* (Mill.) N. E. BROWN (Verbanaceae) (Vale et al., 2002), *Artemisia dracunculus L*. (Asteraceae) (Sayyah et al., 2004), and other aromatic plant species. They present antinociceptive activity when intraperitoneally administered (Do Amaral et al., 2007). Moreover, the action of this monoterpene as a modulator of oxidative stress, also with chemopreventive activity (Manuele et al., 2010) has been reported, reducing skin tumors in animals (Chaudhary et al., 2012). Previous studies have shown that the administration of R - (+) - limonene (0.5 and 1.0%) in mice via inhalation produced anxiolytic effect in an elevated plus maze model (Lima et al., 2013).

R - (+) - limonene, found in many household products, may react with ozone to form free radical species, formaldehyde, and fine and ultra fine particles. Sunil et al. (2007) have shown that young and old mice when exposed to the product of the reaction between limonene and ozone for 3 h show an inflammatory response induction, increasing TNFa, cyclooxygenase-2 and superoxide dismutase in alveolar macrophages. This monoterpene is a potentially sensitizer compound, probably due to the presence of limonene-1,2-epoxide, a product of its oxidation (Karlberg et al., 1999). In contrast, Rolseth et al. (2002) showed that limonene-1,2-epoxide was not responsible for the limonene toxicity when investigated in culture of human pulmonary cells.

Selection of appropriate parameters, the use of a quality control concept and hematological and biochemical analyses using appropriate statistics for the clinical pathology examination of animals are very important for toxicity studies (Matsuzawa et al., 1995).

Research involving prolonged exposure to R - (+) - limonene via inhalation should be performed as limonene has many commercial applications and has been presented as environmental solvents (Rolseth et al., 2002). Therefore, this study aims to evaluate anxiety and toxicity, in accordance with the recommendations of the National Health Surveillance Agency (ANVISA, BRASIL), in rats via inhalation due to the frequent exposure of humans to this substance and its combinations.

MATERIALS AND METHODS

Animals

Wistar rats (*Rattus norvegicus*) (180 – 220 g) were divided into three groups, males (n = 10) for psychopharmacological evaluation and both genders (n = 5) for toxicological evaluation, obtained from the Thomas George Animal Facility of the Research Institute of Drugs and Medicines (IPeFarM-UFPB). All animals were kept until the day of experiments in polypropylene cages with controlled temperature (21 ± 1°C) in 12-h light-dark cycle (light starting at 06:00 am and ending at 06:00 pm). Animals had free access to food (Purina® pellets) and water, up to 60 min before experiments, which were conducted between 06:00 am and noon. All procedures and experimental protocols were approved by the Ethics Research Committee on Animal Use of the UFPB Biotechnology Center (CEUA-CeBiotec) (0606/11).

Drugs and treatments

R - (+) - limonene (97% purity) was purchased from company Dierberger Óleos Essenciais SA, Barra Bonita, Brazil and diluted with Ethoxylated Sorbitan Monooleate (Tween 80 [VETEC, Brazil], 0.2% v / v in distilled water) to prepare R - (+) - limonene (5.0%) emulsions min before the experiment. Control animals received 0.2% Tween 80 via inhalation.

Inhalation apparatus

The inhalation apparatus consisted of an acrylic box (36 × 30 × 29 cm) with a stainless steel grid at its bottom on which animals were individually placed. The front and rear panels contained four holes (each with 2 cm in diameter) with cotton balls embedded with the drug, containing 1 mL per unit of the respective substance (saline or R - (+) - limonene emulsion). The box ceiling contained 30 small holes for ventilation. Based on studies by Lima et al. (2013), the duration of drug exposure (limonene or saline) was 30 min. After each exposure session, the apparatus was cleaned and the cotton ball containing R - (+) - limonene was replaced to maintain the drug concentration in the apparatus.

Elevated plus maze test (EPM)

Three groups of 10 animals were treated with R - (+) - limonene at concentrations of 1.0%, 2.5% or 5.0% via inhalation, while the control group was treated with vehicle. After treatment with R - (+) - limonene or vehicle, animals were individually placed on the central platform facing one of the open arms of the maze, and the number of entries and time spent in each type of arm were recorded for a period of 5 min. Entry into the arm was also considered when the animal was found with all four paws within its limits (Pellow et al., 1985; Bradley et al., 2007; Grundmann et al., 2007).

Repeated dose toxicity (30 days)

Repeated dose toxicity studies were performed using animals of both genders divided into 2 groups (control and treated with 5%).

Each animal was exposed for a period of 30 min at a concentration established once a day. The total period of this protocol was 30 days. At the end of the experiment, animals were euthanized according to criteria established and blood was collected for the analysis of biochemical and hematological parameters.

Laboratory blood analysis

Sample collection was performed by bleeding of the brachial plexus and blood was collected in tubes with ethylenediamine tetraacetic acid anticoagulant (EDTA) to determine hematological parameters and in tubes with separator gel - Microtainer Becton Dickson®. Tubes were centrifuged for 5 min at 3500 rpm to obtain serum for the determination of biochemical parameters.

Hematological parameters

Hematological analysis consisted of the study of red (erythrogram) and white series (WBC) and platelet count. Erythrogram consisted of red blood cell counts, hematocrit and hemoglobin determination, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). In the WBC count, overall white cell count and counting of cell differentiation were made.

Biochemical parameters

Biochemical analyses were performed on serum samples. Dosages of glucose, urea, total cholesterol, triglycerides, uric acid, creatine kinase, albumin, amylase (enzymatic method), creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), alkaline phosphatase (kinetic method) and total proteins (biuret method) were performed in automated biochemical analyzer COBAS MIRA PLUS® - ROCHE.

Statistical analysis

Data obtained for psychopharmacological tests were evaluated using the Kruskal-Wallis test followed by Dunns test. Toxicological data were treated with the "t" Student / unpaired Mann-Whitney test. These data were analyzed using GraphPad Prism software (version 5.0), with experimental group being compared with the control group. (*) P-value <0.05 was considered statistically significant.

RESULTS

Evaluation of anxiety

The animals treated with doses of 1.0, 2.5 and 5.0% showed significant differences when evaluated in the SEM. The three concentrations showed an increase in the number of entries into open arms. However, only 5% concentration showed a statistically significant increase residence time in the open arms (Figure 2).

Evaluation of repeated dose toxicity

Hematological parameters

The repeated dose toxicity test in which animals were exposed for 30 min/day for 30 days showed that some hematological parameters were changed significantly in both gender (Table 1). There was a decrease in erythrocytes, hemoglobin and hematocrit and an increase in VCM, MHCM and HCM.

Biochemical parameters

The biochemical analyses showed a statistically significant increase in glucose, AST and LDH in in both sexes treated with R - (+) - limonene, as compared to the control group (Table 2).

DISCUSSION

Evaluation of the anxiolytic effect of R - (+) - limonene in rats was examined using EPM (Figure 2). The three concentrations (1.0, 2.5 and 5.0%) tested in the study were able to increase the number of entries of rats in open arms. The concentration of 2.5% appeared high in the number of entries in the closed arms being significant only when compared this group with the control. However, to statistically analyze the four groups there is no evidence statistical difference.

In other parameter analyzed in EPM, increase in the time of permanence in the open arms at a dose of 5.0% and a decrease of this time in closed arms for the three dose levels (1.0, 2.5 and 5.0%) were observed. These data analyzed altogether show the anxiolytic effect of R - (+) - limonene, and the dose of 5.0% showed this effect when administered via inhalation in rats for 30 min.

Similar results can be found in previous studies, where the administration of R - (+) - limonene in mice at doses of 0.5 and 1.0% exhibited anxiolytic effect. It was also shown that this EO has an effect similar to diazepam when intraperitoneally administered in mice (Lima et al., 2013). This monoterpene is present at high percentages in *Citrus aurantium* EO (97.83%) having anxiolytic activity (Pultrini et al., 2006; Costa et al., 2013.). Furthermore, a product from limonene oxidation, limonene epoxide, also showed anxiolytic characteristics (De Almeida et al., 2012).

Therefore, EO concentration of 5%, considered with anxiolytic effect in a period of 30 min, was chosen for conducting toxicity tests in repeated doses. After treatment for 30 days, the levels of erythrocyte, hemoglobin and hematocrit count significantly reduced in animals exposed to 5.0% R - (+) - limonene as compared to the control group (Table 1).

The reduction on the erythrocyte and hematocrit count level can be suggestive of hemolysis caused by continuous use R - (+) - limonene (5%) via inhalation for both male and female rats, as the EO has irritant characteristics, inducing inflammatory responses (Sunil et al., 2007). However, these changes are within values

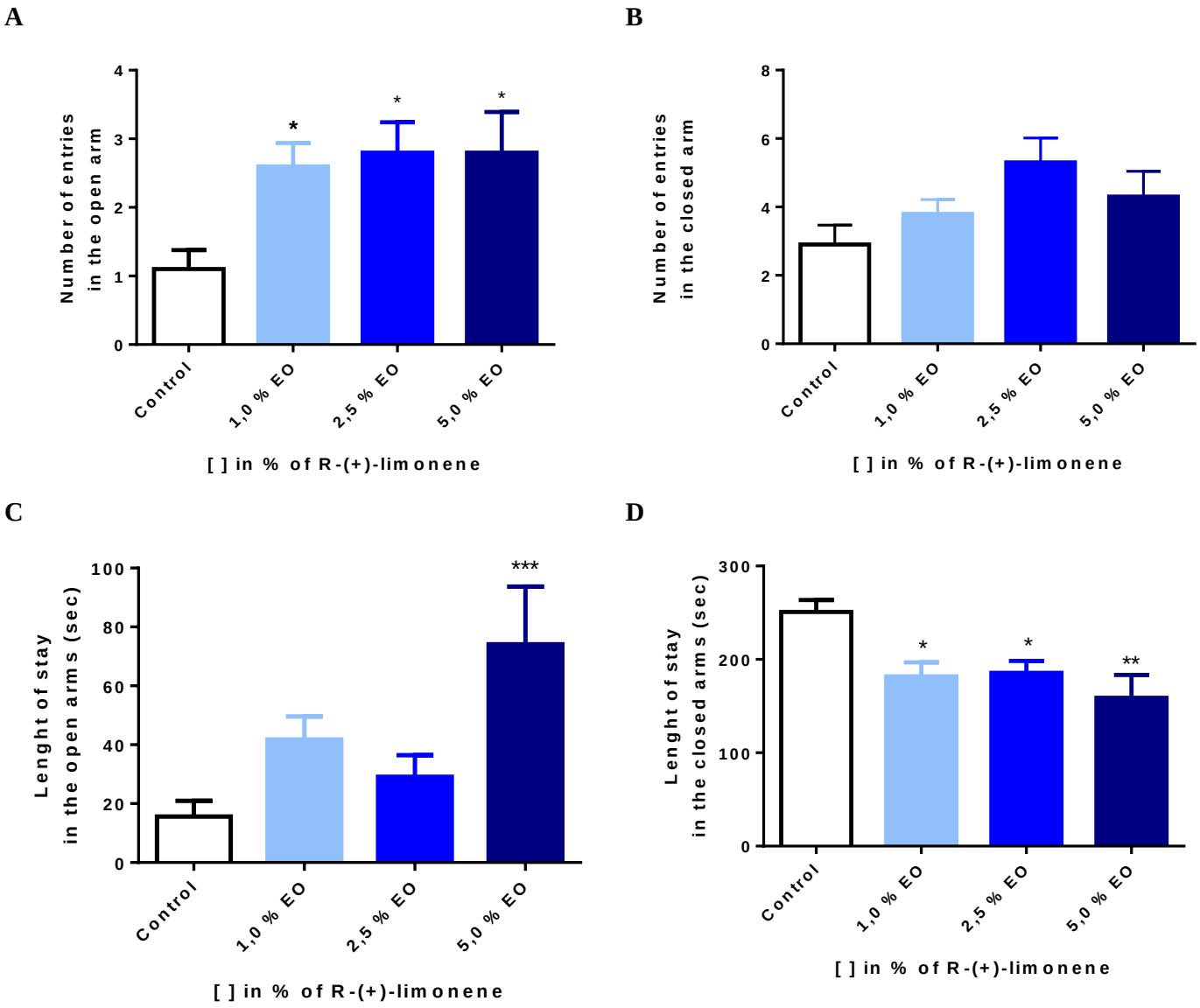

Figure 2. Effects of R-(+)-limonene v.i. (1.0, 2.5 e 5.0%) in time about 30 min the number of entries in the open arms (A) and closed (B) and time spent in the open arms (C) and arms closed (D) of EPM (n=10). Values are expressed as mean ± SEM (n=10). Kruskal-Wallis test followed by Dunns test. *p<0.05, **p<0.01, ***p<0.001.

shown by Lapchik et al. (2009) for red blood cells (5.4 – 8.5 10^6 /mm^3), hemoglobin (11.5 - 16.0 g/dL) and hematocrit (37-49%). Although high, the MCH and MCV values are very close to normal values proposed by Giknis and Clifford (2006). The MCHC values, even high when compared to the control group, are within the normal range (Giknis and Clifford, 2006).

Treatment with R - (+) - limonene in rats with repeated doses induced small changes in the biochemical profile. Some parameters such as glucose, lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) increased significantly when compared to the control group study (Table 2).

Although high when compared to the control group, glucose is within standards in according Giknis and Clifford (2006) where the range for glucose is considered 112 - 176 mg/dL. Already the LDH values showed increased compared to the control group (Table 2). High LDH can cause destruction of the erythrocyte precursor cells in the bone marrow, increasing by up to 50 times the activity of lactate dehydrogenase isoenzymes 1 and 2 (Motta, 2009).

Increases in AST levels are usually linked to liver disorders induced by drugs or hepatitis (Owen et al., 2012). However, toxicity studies with *Citrus limon* and limonene epoxide showed a decrease in AST levels

Table 1. Hematological parameters obtained from the serum of rats treated with 5% R - (+) - limonene inhalation 30 days.

Treatment	Erythrocytes (10^6/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	VCM (μ^3)	HCM ($\mu\mu$g)	MCHC (%)	Leukocytes (10/mm)	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)	Monocyte (%)	Platelets (10/mm)
						Male						
Control	8.7±0.1	14.1±0.2	41.3±0.7	47.8±0.4	16.4±0.2	33.8±0.4	4340±211.2	70.4±1.1	24.2±0.9	1.6±0.3	4.6±0.6	701.2±15.8
R-(+)-limonene (5%)	8.0±0.1*	13.9±0.2	38.9±0.6*	49.6±0.6*	17.3±0.2*	35.3±0.2*	4980±203.5	72.0±1.6	24.8±2.0	1.0±0.2	4.8±0.5	761.2±30.2
						Female						
Control	8.0±0.2	12.9±0.2	39.6±0.5	48.8±1.2	16.7±0.3	34.5±0.2	3020±609.4	77.4±1.8	17.8±1.4	0.4±0.2	4.20±0.8	678.8±57.3
R-(+)-limonene (5%)	7.6±0.1*	13.9±0.3*	37.4±0.5*	52.0±0.7*	17.8±0.1*	35.7±0.2*	4180±640.6	76.8±1.4	17.4±2.5	0.9±0.4	4.2±0.9	673.0±69.1

Values are expressed as mean ± SEM. (n=5). Test "t" de Student/Mann-Whithey. (*) $p < 0.05$. MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration).

Table 2. Biochemical parameters obtained from the serum of rats treated with 5% R - (+) - limonene inhalation 30 days.

Treatment	Glucose (mg/dL)	Urea (mg/dL)	Cholesterol (mg/dL)	Triglycerídes (mg/dL)	Uric acid (mg/dL)	Amylase (U/dL)	AST (U/l)	ALT (U/l)	Alkaline phosphatase (U/l)	LDH (U/l)	Total protein (g/dL)	Albumin (g/dL)
						Male						
Control	119.6±3.3	43.8±2.5	69.0±7.5	111.0±9.6	2.0±0.4	954.4±64.1	130.2±20.2	49.8±3.6	186.0±9.5	2788±467.5	6.9±0.2	3.3±0.1
R-(+)-limonene (5%)	143.8±5.9*	43.0±2.2	56.0±4.7	103.0±18.5	1.5±0.2	912.6±82.3	207.8±20.1*	45.4±3.8	158.6±15.7	4962±482.8*	6.5±0.2	3.0±0.1
						Female						
Control	109.6±4.9	46.0±7.2	54.8±5.2	79.6±9.8	1.6±0.1	677.6±67.1	134.8±16.3	41.6±3.9	104.4±7.7	2837±475.5	6.8±0.2	3.2±0.1
R-(+)-limonene (5%)	124.6±1.0*	42.8±1.8	57.8±3.3	90.4±7.3	2.1±0.2	710.6±16.0	251.6±39.9*	55.2±4.6	107.0±5.3	5599±226.8*	6.5±0.1	3.2±0.1

Values are expressed as mean ± SEM (n=5). Teste "t" de Student/Mann-Whithey. (*) $p < 0.05$. AST (aspartate aminotransferase), ALT (alanine aminotransferase), LDH (lactate dehydrogenase).

when orally administered (Campelo et al., 2013; De Almeida et al., 2014). In dogs and cats, increased AST and LDH levels can be caused by hemolytic diseases and decreased haptoglobin can be caused by the ruptureof erythrocytes inside blood vessels (Fighera, 2007).

Conclusion

Therefore, this study is concluded that the R - (+) - limonene 5% when administered by inhalation for 30 min produces an anxiolytic effect. Through the hematological and biochemical studies, suggest that the R - (+) - limonene in the studied concentration has a low toxicity.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Toxicogenetic biomonitoring of workers to the ionizing radiation

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Occupational exposure to ionizing radiation (IR) can cause systemic acute and chronic effects on human health, including genetic instability that may be etiology of various diseases, including cancer. The aim of this study was to evaluate the possible toxicogenetic changes in haematological and biochemical parameters, and cytogenetic biomarkers (micronuclei and nuclear abnormalities) indicators of mutagenicity and apoptosis, as well as seek their correlation with lifestyle, age and gender. In accordance with the ethical aspects, 45 professionals (technicians and technologists in radiology) occupationally exposed to low doses of IR participated in this study. For control, 45 healthy individuals were not exposed to IR and/or genotoxic chemicals were included. Peripheral blood and oral epithelium samples were used in the toxic evaluations. The results suggested unchanged hematological biomarkers but a significant ($P < 0.05$) increases in the frequency of micronuclei, sprouts, binucleate cells and bridges, as well as karyolysis and karyorrhexis in professional radiology sector. Hepatic and nephritic toxicity were not observed. Without protection, a significant ($P < 0.01$) correlation ($P < 0.05$) was observed between toxicogenetic biomarkers with age, smoking, alcohol consumption, time and place of work. In conclusion, IR may be associated with genetic instability in health diseases, like cancer.

Key words: Ionizing radiation, micronuclei, occupational risk; hematological profile.

INTRODUCTION

Cancer is the second leading cause of death worldwide and is an important public health problem. According to the World Health Organization (WHO), in the coming decades, the impact of cancer in the population will be 80% of the more than 20 million new cases estimated for 2025. In Brazil, in the biennium 2016-2017, about 600,000 new cases of cancer were estimated (INCA, 2015). Among the risk factors, nowadays, radiation and its impacts on human health is a major concern to the etiology of cancer (Samet, 2011). On this occasion, extensive researches are needed in order to diagnose the problems to avoid or at least minimize the deleterious

effects of ionizing radiation (IR) (Pernot et al., 2012).

Notably, ionizing radiation (IR), even at low dose may be harmful and can cause dangerous events in biological systems (Kadhim et al., 2013). An increased dose is always harmful. Sometimes, a particular radiation may act by different mechanistic pathways (Yang et al., 2012). Low doses (0.05 to 0.5 Gy) of IR can cause genomic instability, such as chromosomal alterations and cell death (Kadhim et al., 2013), and can lead to a double failure of DNA and epigenetic alterations in histones (Sasakil et al., 2014). Studies with mass spectrometry based on proteome analysis in human skin model, indicated that after 48 h exposure to 3, 10, and 200 cGy x-rays alter and disrupt 135 proteins, where carboxipeptidases and ubiquitin carboxyl terminus isoenzyme hydrolase were the most sensitive, indicating that radiations at any dose can alter enzymatic proteins (Zhang et al., 2014).

IR have a potential for induced genetic instability in germ and somatic cells, characterized by the production of chromosomal groupings, aneuploidy, micronuclei (MN), gene amplification, mutations in derived and other cells having effects of radiation (Camats et al., 2008). Such changes can be highlighted and monitored by the use of any tests, among them the micronucleus (MN) test is one. It is an easy, economical and reproducible test procedure (Maluf and Riegel, 2011). The MN are formed from the chromosomal fragments or entire chromosomes that are not incorporated into the nucleus of the daughter cell during cell division, they are corpuscles containing DNA without structural connection to the core. The presence of MN and nuclear abnormalities can be considered as a preliminary indication for assessing the mutagenic and/or carcinogenic agents, such as ionizing radiation (Sari-Minodier et al., 2002).

To contribute to the health and prevention of cancer in health professionals occupationally exposed to IR, the present study evaluated the toxicogenetic risks of exposure to the IR using hematological and biochemical parameters in peripheral blood; and the application of cytogenetic MN test in the evaluation of the frequency of MN, nuclear-type abnormalities: binucleate cells, buds, bridges nucleo-plasmatics and apoptosis stages (karyorrhexis and karyolysis) in oral epithelial cells. Secondly, hematological parameters and toxicogenetic biomarkers were correlated to the lifestyle (vegetable consumption, smoking, alcohol consumption, protection of use, age, place of work and working time), age and gender of the participants.

MATERIALS AND METHODS

Ethics and legal aspects

All studies were performed in accordance with Brazilian research guidelines (Law 466/2012, National Council of Health, Brazil) and with the Declaration of Helsinki and all procedures were approved by the Ethics Committee on Human Research, based at the Lutheran University of Brazil (ULBRA, Rio Grande do Sul - CAAE: 38570914.8.0000.5349).

Research subjects

This study was conducted with 45 professionals occupationally exposed to IR including radiology technicians, technologists in radiology and radiologists from two clinics: Diagnostic Unit Imaging (UDI 24 hours, Teresina, Brazil) Lucidio Portela Hospital (Teresina, Brazil) and 45 professionals who do not work in sectors related to diagnostic radiology. All participants answered the health questionnaire recommended by the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) (Carrano and Natarajan, 1998) in order to select the unexposed group as well as for obtaining data on health and lifestyle of the participants to correlate with toxicogenetic parameters.

Haematological and biochemical parameters analysis

Blood samples were collected for hematological and biochemical tests to quantify liver and kidney enzymes such as AST (glutamic-oxalacetic transaminase) and ALT (glutamic pyruvic transaminase), creatinine, urea and alkaline phosphatase (ALP). The tests were performed according to the protocol established by LabtestTM.

Micronucleus (MN) test in oral epithelial cells

Buccal cell samples were obtained by gently rubbing the inside of the cheeks (right and left side) with a cytobrush, which was immersed in 5 ml cold saline (0.9% (w/v) aqueous NaCl) in a conical tube and transported under refrigeration to the Laboratory of Genetic Toxicology, Federal University Piauí (Teresina, Brazil). The samples in saline were centrifuged at 1500 rpm for 10 min and the sedimented cells were then washed with saline (twice) and fixative solution (methanol and glacial acetic acid 3:1) (once) under the same centrifugation conditions. The cell suspension was spread onto a slide with fixative solution and dried at room temperature. The slides were stained with 2% Giemsa solution for 10 min, rinsed in distilled water, and air-dried. Biomarkers of DNA damage (MN), cytokinetic defects (binucleated cells) and cell death (karyorrhexis, pyknotic and karyolytic cells) were scored according to the criteria set by Thomas et al. (2009). For each volunteer, 2000 buccal cells (1000 from each of the duplicate slides) were scored using bright-field optical microscopy at a magnification of 1000. MN were identified taking into consideration in the following conditions: cells with intact main nuclei and cytoplasm; diameter of one-third of the main nucleus; same staining and texture as the main nucleus, and MN was in the same focal plane as the main nucleus. Other anomalies in cells were measured as binucleated, karyolysis, karyorrhexis,and pyknotic cells.

Statistical analysis

Personal characteristics and lifestyle of the groups obtained from the personal health questionnaire were statistically compared by *t*-student's test using the statistical program SPSS 16.0. The relationship between lifestyle characteristics and cytogenetic data were performed by correlation of Spearman's rho. Cellular data

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Table 1. General characteristics of control group and workers exposed to ionizing radiation.

Subject characteristics		Control group (n = 45)	Exposed group (n = 45)
Age[1]		31.73 ± 7.44 (18 - 45)	31.47 ± 7.34 (19- 47)
Gender[2]	Male	68.9 (n=31)	66.7 (n=30)
	Female	31.1 (n=14)	33.3 (n=15)
Alcoholism[2]	Yes	66.7 (n=30)	44.4 (n=20)
	No	33.3 (n=15)	55.5 (n= 25)
Smoking[2]	Yes	22.2 (n= 10)	-
	No	77.8 (n = 35)	100 (n=45)
Prescribed medication use[2]	Yes	22.2 (n=10)	33.3 (n=15)
	No	44.4 (n=20)	66.7 (n=30)
	Not reported	33.4 (n=15)	00.0
Vegetable intake[2]	Yes	20 (n = 9)	13.3 (n=6)
	No	80 (n= 36)	86.7 (n=39)
PPE use[3]	Yes	-	100.0 (n=45)

[1]Mean ± standard deviation; [2]Data in percent form (%); [3]Personal Protective Equipment: coat, mask, apron and boots. Alcoholism was considered as mean of 5 bottles/week. $P < 0.05$; ANOVA, *t*-Student.

on the frequency of micronuclei and nuclear abnormalities between the groups were assessed by the multiple *t*-test with significance in Holm-Sidak method, using the statistical program Graphpad Prism 5.0 (Graphpad Inc., San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Characteristics of population in the study

The characteristics of the population obtained from data collected from the health questionnaire are summarized in Table 1. The mean age of people in the exposed group was 31.47 ± 7.34 years and about 68.9% were male, mostly white (68.9%) and average household 1,097.7 reais (Brazilian currency). With regard to alcohol consumption, 13.3 and and 20,0% of the exposed and unexposed groups answered yes for it, respectively. For smoking, 22.2% of the people of exposed group answered yes and 100% of the members of the unexposed group said that they did not consume cigarettes. Regarding the use of drugs, 66.7% of respondents belonging to the group not exposed and 44.4% of the exposed group reported not using any type of medicine in the last two weeks before the application of the questionnaire. No statistical difference in relation to these characteristics was observed ($P < 0.05$).

Evaluation of haematological an biochemical parameters in health professionals occupational exposure to IR

Hematological (red and,white series and platelets) and biochemical (AST, ALT, creatinine, urea and ALP) were seen within the normal reference standards. Then, no signs of hematological changes and liver and kidney toxicity were detected (Table 2).

Genetic instabilities induced by occupational exposure to IR in oral epithelium of health professionals

The risk assessment for occupational exposure to hazards has social importance and for worker health (Montano, 2014). Epidemiological studies show an association between IR and cancer, like brain cancers (Smoll et al., 2016). High and low doses of IR induced circulatory diseases are indicative of risks following occupational exposure to mortality risk, as well as with the cancer-inducing ability (Little et al., 2012). The MN test is well founded to establish the risk of DNA damage and studies show a strong association between IR, increased frequency of MN and susceptibility to cancer (Bolognesi et al., 2014).

Workers exposed occupationally at IR in Radiological Diagnostics Clinics in Teresina- -Piauí, showed genetic instability in oral epithelium cells ($P < 0.001$) since it occurred increase in MN frequencies, nuclear bud (NB), binucleate cells (BN) and nucleoplasmatic bridges (NP), when compared individuals not exposed to IR (Figure 1). Previously cytogenetic studies reported impacts in mammalian cells exposed to 0.5-3 Gy with presence of DNA damage confirmed by chromosomal aberrations and MN, which are coming from breaks and loss of chromosomes, suggesting a genetic instability (Plamadeala et al., 2015).

Oral epithelium is important tool for toxicological

Table 2. Hematologic and biochemistry parameters of workers occupationally exposed to ionizing radiation.

Parameter	Reference range		Control group		Exposed group	
Red line	**Man**	**Woman**	**Man**	**Woman**	**Man**	**Woman**
Red cell Count/mm^3	4.3 – 5.7	3.9 – 5.0	4.9 ± 0.2	4.6 ± 0.2	4.6 ± 0.2	4.5 ± 0.3
Haemoglobin g/dL	13 – 17.5	12 – 15	15.5 ± 1.0	13.7 ± 1.2	14.1 ± 1.1	13.2 ± 1.0
Haematocrit (%)	38 – 50	36 – 44.5	45.6 ± 2.4	42.2 ± 2.1	43.0 ± 2.9	40.3 ± 3.0
White line						
White cell Count/mm^3	4.000		5588 ± 1000	5148 ± 1065	5221 ± 990	5473 ± 1100
Neutrophils/mm^3	45 – 69		58 ± 5.8	56.4 ± 5.3	5221 ± 990	5473 ± 1100
Lymphocytes/mm^3	20 – 47		37 ± 4.7	39.5 ± 4.4	37.7 ± 5.4	36.3 ± 3.5
Monocytes/mm^3	1 – 10		3 ± 1.2	2.4 ± 1.3	3.0 ± 1.4	2.8 ±1.0
Eosinophils/mm^3	0 – 5		1.6 ± 1.0	2.2 ± 1.2	1.9 ± 1.1	1.2 ± 0.8
Basophils/mm^3	0 – 1		0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Platelet Count	150 – 450 K		281 ± 60	281 ± 55	270 ± 42	299 ± 84
Biochemistry parameters						
AST (U/L)	37	31	31.4 ± 7.1	23.9 ± 8.4	21.4 ± 5.0	19.2 ± 3.9
ALT (U/L)	42	32	33.5 ± 10.1	26.2 ± 15.3	20.8 ± 12.0	14.9 ± 5.3
Creatinine (mg/dl)	0.4 – 1.4		0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
Urea (mg/dl)	15 – 40		29.8 ± 4.8	34.8 ± 11.0	36.4 ± 8.8	34.0 ± 5.3
Alkaline phosphatese (U/L)	27 – 100		42.3 ± 11.3	69.7 ± 22.9	76.5 ± 18.6	73.4 ± 18.9

studies, especially due to its rapid division. MN and other nuclear abnormalities such as sprouts and bridges are considered biomarkers for genotoxic damage and chromosomal instability. MN are fragments of chromosomes or loss of chromosomes during anaphase of cell division due to the poor chromosome segregation as a result of hipometilations in repeated sequences of the centromeric regions (Fenech et al., 2011).

MN, extra nuclear bodies, may be observed in oral exfoliated mucosa cells, as a biomarker for genotoxicity and carcinogenicity. The formation mechanism of NBs may be related to the elimination and amplification or DNA repair. The BNs may be indicative of cytokinesis failure at the end of cell division (Sabharwal et al., 2015). The MN formation may be due to the breakage, indicating clastogenic and chromosomes aneugenic agents (Suzuki et al., 2003; Fenech et al., 2011).

Nuclear changes induced by occupational exposure to IR in oral epithelium of health professionals

Exposure to occupational and environmental radiation sources can cause damage and genetic instability due to its effects as carcinogens. Epidemiological studies show an association between IR at low doses (1 to 5 mGy) and development of leukemia. Leukemia is a complex disease that may have etiologies related to the lifestyle and occupational and environmental exposure, but there are also associations between leukemia (Polychronakis et al., 2013) due to genetic instability related to increased single and double strand DNA breaks that can lead to the chromosomal alterations and formation of MN (Saha et al., 2014), as noted in mutagenicity analysis of professionals occupationally exposed to IR in this study. There are also reports that IR can lead to apoptosis, as observed in embryonic neuronscells (Leuraud et al., 2015).

Nuclear abnormalities such as BNs and apoptosis, karyorrhexis (nuclear fragmentation), and karyolysis (nuclear dissolution) are indicative of cytotoxicity were observed in health professionals exposed to IR. Data were statistically significant ($P < 0.05$) when compared with the control group (Figure 2). BNs may be indicative of cytokinesis failure at the end of cell division and cells with condensed chromatin are indicative of apoptosis (karyorrhexis and karyolysis), as well as picnoses (Sabharwal et al., 2015). Exposure to IR can lead to oxidative events, one of the mechanisms of cytotoxicity and apoptosis, due to the fact that these have direct action on the macromolecules *via* radiolysis product and intracellular modulation of the communication mechanisms of redox system, which induce stress in cells, tissues and mitochondria, led to the toxic effects of radiation depending on the doses (Einor et al., 2016).

IR, even at low doses, induce gene amplification and reactive oxygen species (ROS), for transducing signals for the enzyme superoxide dismutase (SOD), as observed in studies of brain cells from rats submitted to 2, 10 and 50 cGy, with the modulation about 88 signaling molecules which can induce apoptosis (Veeraraghavan et al., 2011). The accidental exposure to IR in doses 0 to 8 Gy in rats cause damage to brain cells that can generate neurodegenerative and cognitive complications with an induction of p53 and a decreased expression of proliferating cell nuclear

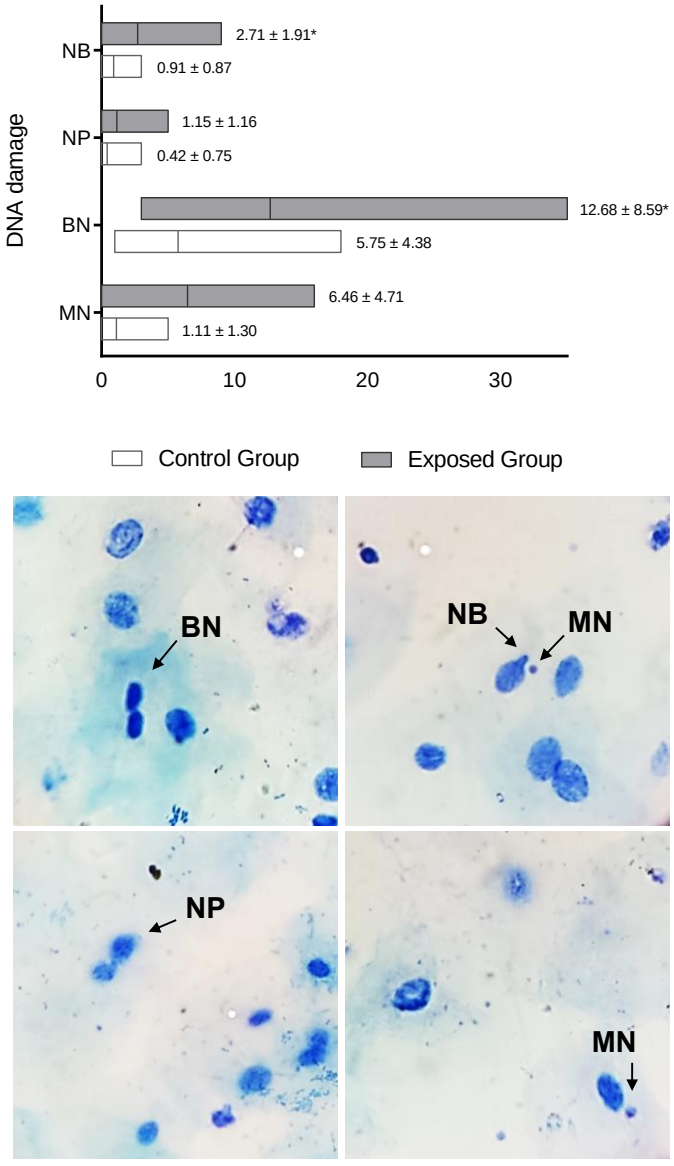

Figure 1. DNA damage assessed by cytogenetic biomarkers front of occupational exposure to ionizing radiation. NB: Nuclear buds; NP: nucleoplasmic bridges; BN: binucleated cells; MN: micronuclei. Means ± standard deviation, multiple t test and Holm- Sidak method, *P <0.05 compared to control group.

antigen (PCNA) and cell proliferation. The reduction of PCNA may be associated with the induction checks during the cell cycle (Chen et al., 2015).

Ionizing radiations have adverse responses in human cells, including as discussed previously in apoptosis, necrosis, premature senescence stress, autophagy and endoploidy. The p21 and p53 genes are important for cell responses front to the DNA damage processes and apoptosis induction (Mirzayans et al., 2013). Apoptosis has an important role in the homeostasis as well regulation of apoptosis levels related to the prevention of diseases and may be an indicative of genotoxic stress. Several signaling pathways regulate apoptosis among them involving the BCL-2 family protein, and inhibitors of apoptosis (IAP) (Hassan et al. 2014). The effects of IR involve endogenous signaling events that culminate in oxidative damage to DNA, lipids, proteins and many metabolites, in addition to changes in gene expression, metabolism and epigenetic factors (Reisz et al., 2014). It is well established that IR induce instability in chromosomes and have effects on activation and inactivation of DNA repair mechanisms, induce oxidative damage, but is not yet well established role of IR on telomeric proteins, which are considered to be protective of genome and related to carcinogenesis (Shim et al., 2014).

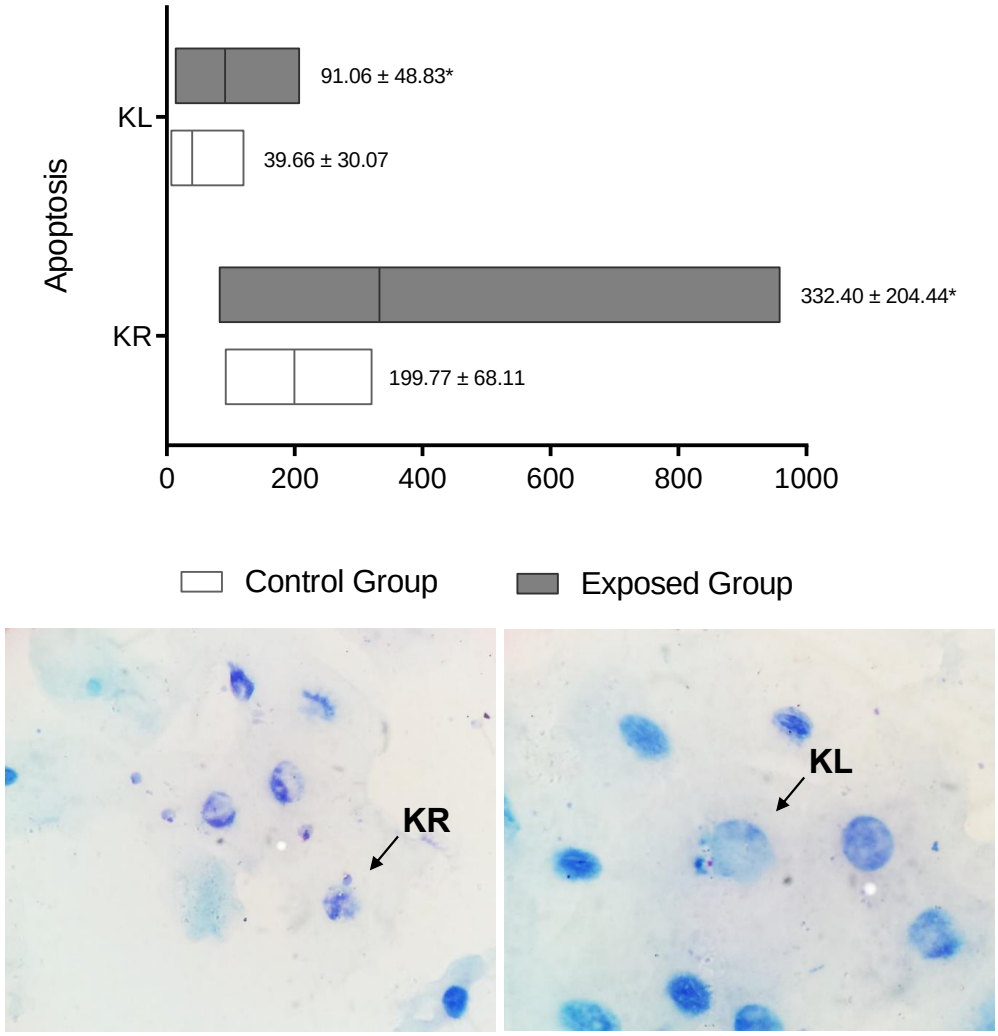

Figure 2. Apoptotic DNA fragmentation in buccal mucosa cells from workers exposed to ionizing radiation and control group through Micronucleus test. ANOVA. Non-parametric Mann-Whitney U test. *P <0.05 compared to the control group.

Numerous effects of radiation have been identified in association with mutations and genomic instability (breaks and actions in repair genes), angiogenesis (vascularisation and hypoxia), apoptosis (changes in p53), proliferative signaling (EGCR and TGF-α), suppression of cell cycle (ATM, p53 lock), energy dysregulation (HIF, c-MyC, glycolytic pathways inhibitors), tumor promotion and inflammation (p53 and ROS) and activation and inactivation of metastases (hypoxia, lactate) (Boss et al., 2014). Given the risks of cancer, studies suggest biological dosimeters guided by genetic instability mechanisms, is noted in summary in Table 3, especially for chromo-somal aberrations that may be markers for evaluation of dose and biological effects induced by IR (Higueras et al., 2015). Numerous effects of radiation (Table 3) have been identified in association with mutations and genomic instability (breaks and actions in repair genes), angiogenesis (vascularization and hypoxia), apoptosis (changes in p53), proliferative signaling (EGCR and TGF-α), suppression of cell cycle (ATM, locking p53), energy deregulation (HIF, c-MyC, glycolytic pathways inhibitors), tumor promotion and inflammation (p53 and ROS) and activation and inactivation of metastases (hypoxia, lactate) (Boss et al., 2014; Lee et al., 2014). Relation among age, gender, DNA damage and cytotoxicity.

Populations occupationally exposed to chemical and physical genotoxic agents are being carried out with the application of toxicogenetic tests. However, the understanding of the influence of factors related is necessary to age, gender and lifestyle and disease in relation to MN rates (Bonassi et al., 2011), as well as other nuclear abnormalities (Holland et al., 2008).

To evaluate the influence of age for MN induction, cells with karyorrhexis (indicative of apoptosis) and the mean ages were compared with those toxicogenetic biomarkers. The mean of MN of different age intervals

Table 3. Possible action mechanisms of ionizing radiations in workers occupationally exposed.

Mechanisms	Dose/Biological sample	References
Chromosome damage, dicentric chromosomes, nucleoplasmic bridges, chromosomal rings, fragments	Low dose (0-100 mGy)/ Blood	Manning and Rothkamm (2013)
Chromosomal aberrations and micronuclei	0.5-5.0 Gy/Blood	Liu et al. (2009)
Chromosomal aberrations, micronuclei and apoptosis	0.325 mGy/Buccal mucosa cells	Arora et al. (2014)
Changes in DNA, lipids and expression of proteins and epigenetic factors	15-30 mGy and 0.01-0.15 mGy/Blood	Reisz et al. (2014)
Dicentric chromosomes	0-5 Gy/Peripheral blood	Al-Hadyan et al. (2014)
Metabolic and immune dysfunction	0.5-5 Gy/Peripheral blood	Lee et al. (2014)
Activation and inactivation of telomeric proteins	High and low doses	Shim et al. (2014)
Mutations and genomic instability. Angiogenesis, apoptosis, proliferation, energy imbalance, inflammation and metastases	High and low doses	Boss et al. (2014)

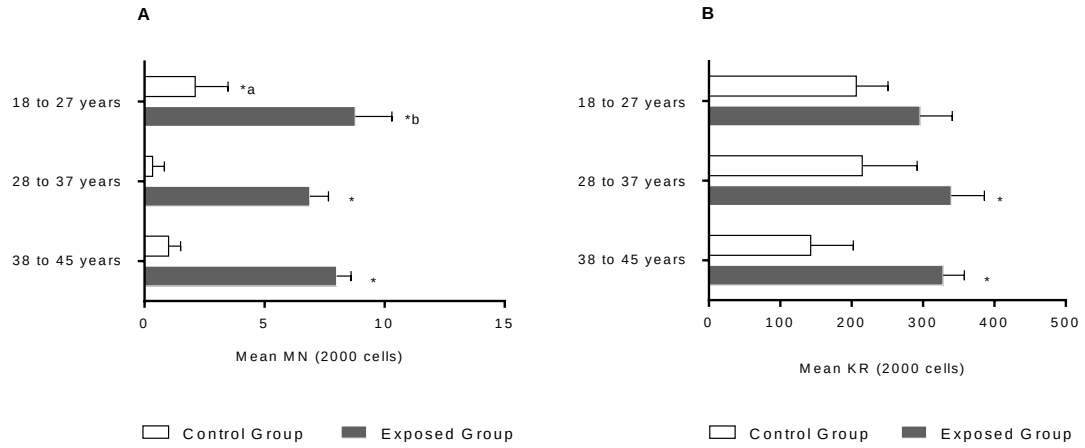

Figure 3. Mutagenicity (A) and apoptotic DNA fragmentation (B) according to tertis of age in buccal mucosa cells from workes exposed to ionizing radiation and control group. ANOVA, Bonferroni's multiple comparison test. *P <0.05 compared to control group. a,b compared to age intervals of 28 to 37 and 38 to 45 years. MN: Micronuclei; KR: Karyorrhexis.

of exposed workers was significantly higher compared to the unexposed group. Workers exposed that age range of 18 to 27 years had more production of the MN when compared to other age ranges (Figure 3A). For karyorrhexis, the data were significant only for the group unexposed to X-rays (Figure 3B).

MN test in relation to the age of the exposed workers showed that in all ages, the exposed workers are more susceptible to IR than the unexposed

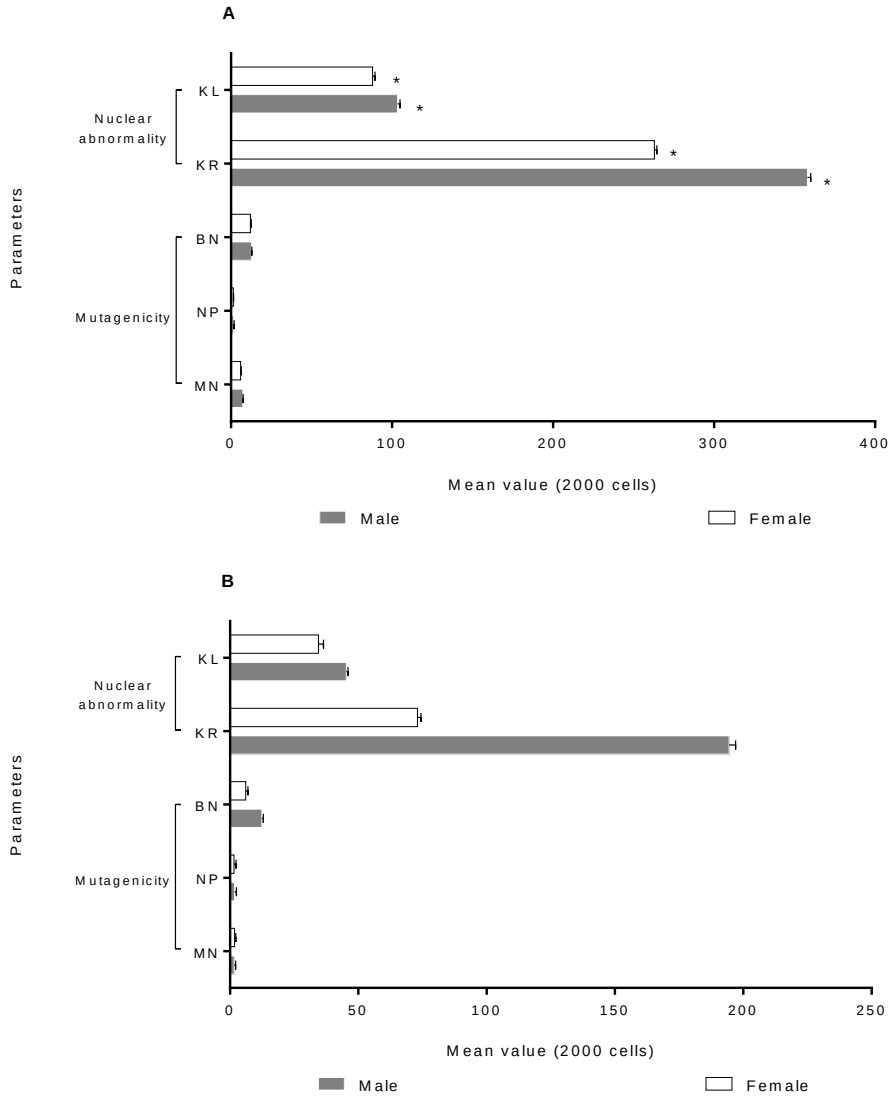

Figure 4. Comparative study of cytogenetic biomarkers in relation to gender. ANOVA, MannWhitney U test. A: Exposed group; B: control group; *P <0.05 compared group B; NP: Nucleoplasmic bridges; NB: nuclear buds; BN: binucleated cells; MN: micronuclei; KR: Karyorrhexis; KL: Karyolysis.

group. These data are in agreement with Maluf and Erdtmann (2001), who evaluated the presence of cellular with Maluf and Erdtmann (2001), who evaluated abnormalities in workers exposed to radiation than the consumption of oxide ethylene and cytostatic agents. In comparison to the age range, the age of professionals between 18 and 27 years showed more significant levels than the other groups with respect to the mutagenicity, and the range of 38 to 45 showed more significant indices with respect to apoptotic changes. These data differ from the data presented by Ladeira et al. (2011), where the evaluation of abnormalities found in the MN test showed no statistical difference in age and sex. However, Jha and Sharma (1991) revelaed an apoptosis and nuclear abnormalities in workers age between 35 to 50 years exposured to IR.

Regarding gender, it was observed that only karyorrhexis level is statistically significant with a greater predisposition to nuclear fragmentation in males. On the other hand, Fenech and Bonassi (2011) showed that the changes in MN test are always higher in women than the men in all age groups. This occurs due to the hormonal cycle in women and especially the low intake of some micronutrients such as vitamins, folic acid and ions. Although, there were no statistically significant differences (P >0.05) for cytogenetic biomarkers tested in relation to gender differences in groups exposed to IR in the unexposed group. In this study, we observed differences only karyorrhexis when assessed alone (P <0.05, Figure 4).

Correlation between lifestyle factors with cytogenetic biomarkers

Genomic damage has great important in the etiological

Table 4. Correlations between lifestyle and cytogenetic damages.

Parameter	Sperman's rho	P value
Life style *vs.* biomarkers	-	-
Smoking *vs.* Micronuclei	0.426	0.002*
Alcoholism *vs.* Micronuclei	0.521	0.000*
Age *vs.* Nucleoplasmic bridges	0.313	0.036*
Age *vs.* Karyolysis	0.382	0.010*
Time of work *vs.* Karyolysis	0.319	0.330
Time of work *vs.* Binucleated cells	0.416	0.005*
Work place *vs.* Karyorrhexis	0.424	0.004*

Sperman's correlation. * P < 0.05.

analysis for the development of degenerative diseases. Radiation and the use of chemicals are needed in different therapies, but also micronutrient deficiencies, lifestyle and other genetic factors are needed in biomonitoring, diagnosis and treatment of diseases in the evaluation of genetic damage (Tolbert et al., 1992).

Herein, after statistical Spearman's Herein, after statistical, positive correlations were observed between smoking and alcohol consumption with MN, age bridges and karyolysis and working time with karyolysis and binucleate cells in oral epithelium (Table 4).

Cytogenetic damage shown in this study did not show positive correlations with age, smoking, alcohol consumption, and genetic diseases, with exposure to IR. Hartmann et al. (1998) also demonstrated an insignificant relationship between smokers/nonsmokers and DNA damage. However, epidemiological studies show that smoking induces DNA damage, and may cause lung cancer (Sram, 1998). To be noted that age-related increased in the risk of aneuploidy and non-disjunction mitotic, changes in chromosomes with a reduced DNA repair are suggested by Migliore et al. (1991), despite of a controversial talk by Betti et al. (1994). These different responses can be related to the size of the samples, as well as varied and individual susceptibility of the population under study.

CONCLUSION

Occupational exposure of ionizing radiation to the workers caused genetic instability in a independent way of age and gender, induce micronuclei, bridges, buds and binucleate cells. However, an unchanged haematological parameters and hepatic as well as nephritic function were observed. A correlation with risk factors related to lifestyle (smoking and drinking) and IR was established. Our study demonstrates biomonitoring of genetic risks, applying mutagenicity biomarkers and nuclear abnormalities can be alternative tools for the diagnosis and prevention radiation-induced health hazards.

Conflict of Interests

The authors have not declared any conflict of interests.

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