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Original Article

TOXICOLOGICAL EFFECT OF ACUTE EXPOSURE OF SULFUR DIOXIDE ON BUCCAL MUCOSA OF MUS MUSCULUS

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ABSTRACT

Exposure to toxic gas emissions from industries or other sources represents an important cause of negative effects on the health of living organisms. The objective of this work is to detect the possible cytotoxic or genotoxic effect on mice exposed to SO2 gases, so we implemented a system with an airtight chamber where BALB/c mice were located, distributed into control (air) and treatment (air-SO2) groups exposed to a constant flow of air-SO2 at concentrations of 0, 200, 250, 300 and 350 µg/m3 of SO2 at 27°C continuous in 72 hours. Through microscopic observation (1000X), oral mucosa epithelial cells stained with May-Grunwald Giemsa were examined. A statistical analysis was performed using ANOVA with Tukey's post hoc test and the SPSS 21.0 program. The results (mean ± SD) showed significant differences between the different treatment groups respect to the control. It was also an increase in the percentage of cells with cytotoxicity (18.09 ± 4.43 and 45.85 ± 2.74 of 250 and 300 µg/m3 of SO2 respectively vs. 11.65 ± 4.45 of the control) and genotoxicity was observed (16.07 ± 6.25 and 18.03 ± 1.34 of 250 and 300 µg/m3 of SO2 vs. 10.85 ± 3.50 of the control). Therefore, it is concluded that increase cytotoxic and genotoxic index at these SO2 values.

KEYWORDS Sulfur dioxide, Oral Epithelium, Cytotoxicity; Genotoxicity & Environmental Pollution

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INTRODUCTION

Atmospheric pollution is the presence of harmful or undesirable substances from natural and/or artificial sources that in sufficient quantity can cause discomfort or health risks of any living being (Martínez and Díaz, 2004). Sulphur dioxide (SO2) is a non-explosive, non-flammable, irritant colorless gas and is the main Sulphur-derived pollutant found naturally in the vicinity of volcanoes; it is also generated as a result of the combustion of coal, oil, diesel, natural gas and mineral processing, for years it has been known to generate acid rain by transforming it into sulfuric acid in the atmosphere (Kampa & Castañas, 2008).

SO2 intoxication has been continuously addressed from different points of view; for example, it was shown that exposing mice to SO2, the activity of oxidase sulfide was inhibited, leading to death in short periods of time; this is attributed to the non-transformation of SO2, thus dominating the toxic activity of this gas (Cohen, 2017). In another study, after a short time of exposure to gas, it even caused irritation even in the tissues of the larynx; there is no information on the long-term health problems caused by this exposure (Sarnat, 2006).

Another important study shows that inhaling SO2 by people operating in laboratories are negative affected at a cellular level. In their oral and nasal mucous membranes, they developed cells with micronuclei (MN) that do not allow normal cell development. The population of normal cells declines at the expense of abnormal cells with micronuclei (Sandivar et al., 2014). It has also been determined that oxidative stress induced by constant

exposure to SO2 has a negative effect on the respiratory and cardiac system in vertebrates because it causes the destruction of their cells by altering biological structures membrane lipids, proteins and even their DNA (Romero-Calderon, 2017) on the other hand, it has been linked that oxidative stress is related to cell aging and some pathophysiological processes such as cardiovascular diseases, neurodegenerative diseases and certain forms of cancer (Venereo, 2002; Avello and Suwalsky, 2006).

Whit respect to Peru, the presence of SO2 in our atmosphere is becoming a problem that can get out of control, this attributed to the Ministry of the Environment following DS 003-2017 MINAM established new emission parameters through the Environmental Quality Standards (ECA) for air, according to this Supreme Decree, in the case of SO2 in air was increased from 80 µg/m3 to 250 µg/m3, in addition in the air quality report indicates that the cities of Cerro de Pasco, Cusco, Chachapoyas and Huancayo have high levels of SO2 concentration, which highlights the city of Oroya (Cerro de Pasco) since in this city during 2013 extremely high values of up to 577 µg/m3 were detected exceeding the maximum permissible limit according to our regulations of 250 µg/m3, which were directly associated with the activity in the area (MINAM, 2014). At the internationally level, the EPA establishes exposure limits to said maximum daily gas for one hour at 75 ppb or for 3 hours at a 0.5 ppm (EPA, 2017).

Because of this, the main objective of this research was to quantitatively evaluate cytotoxicity and genotoxicity at the mouse oral epithelium level in order to refer by extrapolation to these results and to propose preventive measures by irrigation which involves exposure of SO2 by inhalation by humans.

MATERIALS & METHODS

Test Agents and Reference Items

The source of the air-SO2 mixture used in the experiments was obtained in the USA through PRAXAIR-Lima, the mixture supplied was 10m3 in 10 ppm Primary Standard with certified concentration of 10.7 ppm. The dilution source used was medicinal quality air packaged in 6.0m3 vessels and was obtained from Tecnogas S.A.-Lima, pressure regulators were installed in both vessels to control flows. The experimentation cockpit consists of a cover made of cubic-shaped acrylic (Figure 1). To avoid excess pressure provided by gas cylinders, each operated with a pressure regulator.

Animals and Housing Conditions

A total of 25 young adult male Balb/C mice (8 – 12 weeks old at delivery) were obtained from the certified animal room of the National Institute of Health (INS) of Lima. They were housed in an air-conditioned animal room of the Faculty of Biological Sciences of UNMSM. Lighting consisted of a controlled 14 hours light and 10 hours darkness cycle. Animal were given free access to standard diet and drinking water, except during the exposure periods. Prior start of the study, the mice were acclimatized for at least 7 days to the housing conditions in the room animal of UNMSM. The management, care and experimentation of mice were carried out in accordance with the Guide for care and use of laboratory animals (NRC, 2011) and with the bioethics standards established by the FONDECYT and CONICYT Bioethics Advisory Committee (FONDECYT, 2009).

Animal Treatment

The mice were randomly assigned to five treatment groups. The first group, negative control (CN) was exposed only to the air mixture during 72 hours; TI, TII, TIII and TIV groups (200, 250, 300 and 350 µg/m3 SO2) were continuously exposed to the air – SO2 mixture at the different concentrations during 72 hours.

Micronucleus Test

Buccal Mucosa Cell Preparation

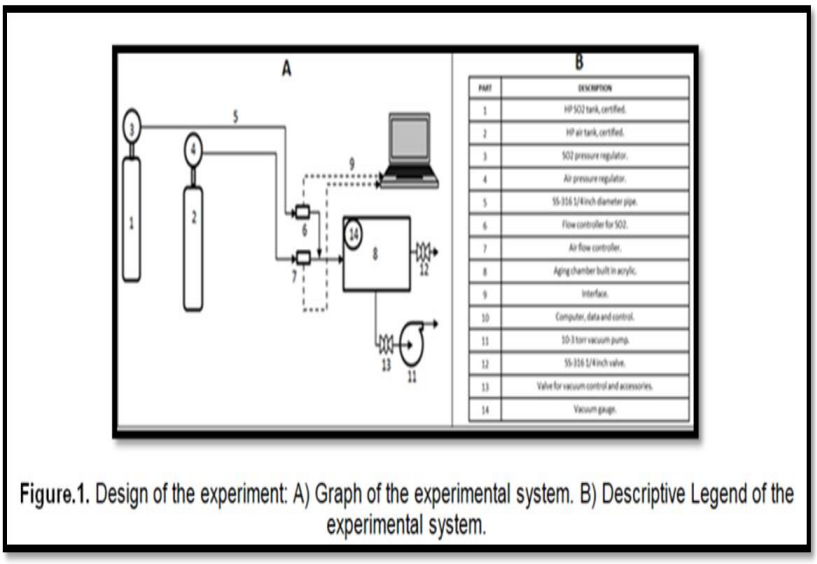

Figure.1. Design of the experiment: A) Graph of the experimental system. B) Descriptive Legend of the experimental system.

Figure 1

The previously anesthetized animals were slaughtered and by sterile swabs proceeded to the flaking of surface cells of the mucosa of the oral epithelium. The end of each swab was cut and placed in sterilized vials and subsequently homogenized by means at a vortex (LAB-LINE INSTRUMENT) in saline phosphate buffer (PBS) at 37°C for immediate processing. Vials with epithelial cells were centrifuged for 20 minutes at 1200 rpm and most of the supernatant was discarded. The cell pellet was re-suspended in 1.5 mL of fresh PBS (phosphate buffered saline). Four more washes were performed, added 1.5 mL of buffer (0.1M EDTA, 0.01M tris-HCl, 0.02M NaCl pH-7) and centrifuged for 10 minutes at 1250 rpm. With the result of each vial per individual the smear was performed on two slides. Finally, the slides were left to dry on a stove tempered at 37°C for 15 minutes.

Slide Fixing, Coloring and Sealing

Each sheet was fixed with absolute methanol at 8°C for 2 minutes; the sheets were then left to dry under ambient temperature conditions for 1 hour. The coloring was performed with May-Grunwald Giemsa 5% prepared in PBS pH 7.2 for 10 minutes. After that time the excess dye was removed with running tap water and then allowed to dry at the environment. Finally, the slides were permanently encoded and fixed with sealant, for later photographic visualization and analysis.

Cytotoxicity Analytic

There are nuclear abnormalities in cells that are indicative of cytotoxic damage. All of these correspond to different biological processes (Tolbert et al., 1991). The most common are the following: Pycnotic nucleus, degeneration where the nucleus undergoes a retraction or decrease in size due to the condensation of its chromatin. At high levels it is a response of injury or cell damage. Cariorrexis nucleus or disintegration, it is the nuclear degeneration where it disappears, and the

genetic material is represented in small irregularly shaped particles. Kariolysis nucleus or nuclear dissolution, where the nuclear membrane is conserved ant the chromatin solution is expressed as loss of color.

200 cells under a clear field optical microscope were analyzed at a 1000X, distinguishing between normal cells and cells with some type of nuclear aberrations per individual, with the frequency recorded in percentage.

Genotoxicity Analysis

200 cells in a clear field optical microscope were analyzed at a 1000X, distinguishing between normal cells and cells with micronuclei, i.e. cells that have small nuclei that are located next to the cell nucleus by each individual, the frequency in percentage.

Statistical Analysis

The data were expressed as an average of standard deviation (SD). The normality of the data was analyzed by the Shapiro –Wilk test and statistical analyses were performed using ANOVA and the Tukey post-hoc test. P values less than 0.05 ($p<0.05$) were considered statistically significant. In addition, Pearson's correlation analysis was performed to determine the dose response ratio. These statistics were implemented in SPSS 21.0 for Windows.

RESULTS

The first micronucleus test in exfoliation cells as an indicative of genotoxicity was carried out at the beginning of the 80 by Stich et al. (1982). They used oral mucosa cell to assess genotoxic damage due to tobacco exposure (Stich et 1982 & Stich and Rosin 1983). Unlike other cell types, the epithelium is formed by several cell layers that are exfoliated as they reach the surface, so if genotoxic and cytotoxic damage is found at the level of the surface it is inferred that this damage has occurred at the basal cell level (Schitch & Rosin 1983), so the use of oral epithelial tissue is very convenient for analyzing the effects of cytotoxicity and genotoxicity.

SO2 enters the body through the respiratory tract; next, diluted in saliva by entering the gastrointestinal tract in the form of sulfurous acid (Parmigianino,1983), it is also absorbed into the respiratory tract and then passed into the blood and distributed throughout the body, reaching various tissues including the epithelial, then it is concentrated in the liver, spleen, esophagus and kidneys, it has been found that significant amounts of SO2 are retained in the lungs and trachea of experimental animals. (Sullivan et al., 1992).

When there is a constant exposure to various types of gases, for example: SO2, reactive oxygen species (ROS) are generated in the body (Meng et al., 2005), the balance is broken due to the accumulation of excess ROS in the lung, which is where the gas comes first. The blood collects oxygen from the lungs so that it can be transported to the rest of the body and nourish the cells, but when there is an excess of ROS very apart from fact that the erythrocytes can become affected, those in conditions of power transport oxygen, they also carry ROS to other cells (Mohanty et al., 2014).

In our study, genotoxicity increases in a dose-dependent manner, being significant from the dose of 350 µg/m3 of SO2 (see table 1 and figure 2), while cytotoxicity increases in a dose-dependent manner, being significant from the dose of 300 µg/m3 of SO2 when accounting for total cytotoxicity, but from 250 µg/m3 of SO2 with respect to the percentage of cells with picnosis (see table 2 and figure 3), this is due to the excess of ROS generated as a result of the concentration of SO2 in the atmosphere since it can directly damage the mitochondria, triggering various signaling pathways that generally induce cell death, so that cells with cytotoxic damage evidenced in nuclear aberrations can be observed, such as picnosis

(condensation of the cell chromatin), cariorrexis (nucleus fragmentation) and karyolysis (destruction of the nucleus) (Hare, 2001). The number of cells with micronuclei, genotoxic damage, increase while increasing the concentration of SO2 (see table 1), because ROS alters biological structures such as proteins and DNA (Fedde and Kuhlmann, 1979).

Table 1: Effect of SO2 on the Variation of the Percentage of Cells with Genotoxicity According to the Treatment Groups. Mean ± SD. The Significant Differences (P <0.05) with Respect to the Control Group are Represented by an Asterisk

Treatment Groups	Genotoxicity (%)	Cells with one MN (%)	Cells with two MN (%)
Control group	10.85 ± 3.50	10.02 ± 2.71	0.83 ± 0.96
200 µg/m³	15.14 ± 0.94	10.71 ± 2.25	4.43 ± 0.66*
250 µg/m³	16.07 ± 6.25	13.27 ± 5.65	2.80 ± 0.89
300 µg/m³	18.03 ± 1.34	11.57 ± 0.69	6.46 ± 1.12*
350 µg/m³	25.63 ± 2.89*	19.87 ± 2.94*	5.76 ± 1.17*

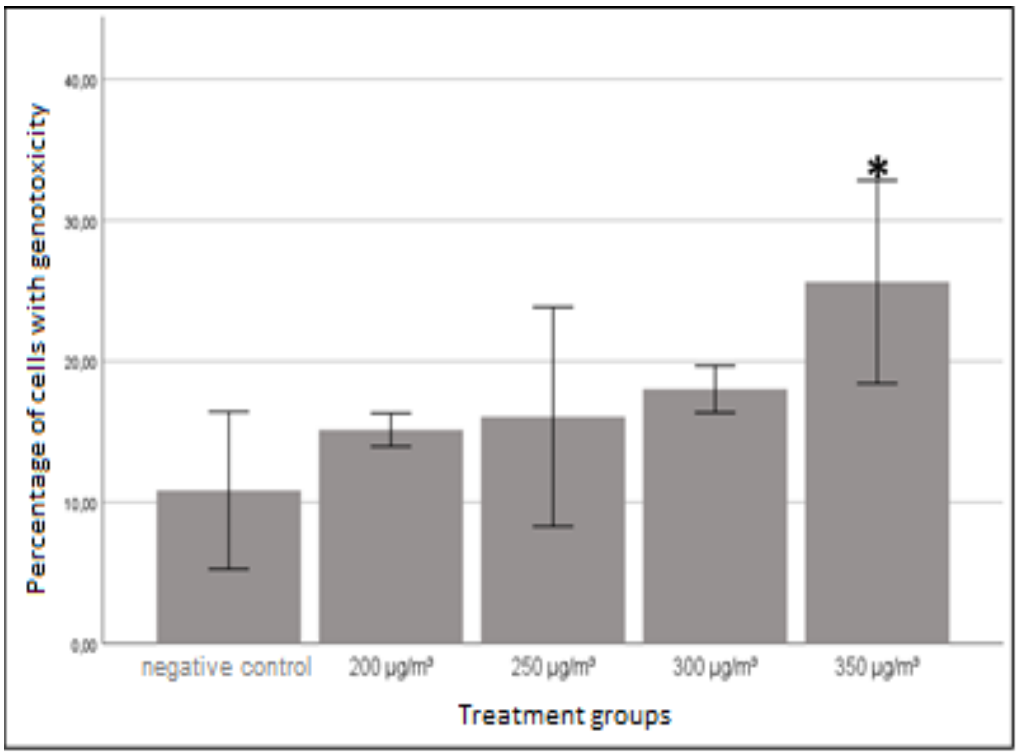

Figure 2: Effect of Sulfur Dioxide (SO2) on the Variation of the Percentage of Cells with Genotoxicity According to the Treatment Groups. The Bars Represent Mean ± DS. The Significant Differences (P <0.05) Respect to the Control Group are Represented by an Asterisk.

Table 2: Effect of SO2 on the Variation of the Percenage of Cells with Cytotoxicity, Cells in Pynosis, Cells in Cariolexis and Cells with Karyolysis According to the Treatment Groups. Mean ± SD. The Significant Differences (P <0.05) with Respect to the Control Group are Represented by an Asterisk

Treatment Groups	Citotoxicity (%)	Pynosis (%)	Cariolexis (%)
Control Group	13.51 ± 4.09	3.69 ± 0.51	6.15 ± 5.59
200 µg/m³	13.59 ± 1.00	5.16 ± 2.79	3.37 ± 0.88
250 µg/m³	18.09 ± 4.43	10.83 ± 2.12*	2.08 ± 1.47
300 µg/m³	45.85 ± 2.74*	19.64 ± 4.82*	6.58 ± 1.15
350 µg/m³	48.72 ± 1.23*	13.05 ± 0.14*	10.92 ± 0.49

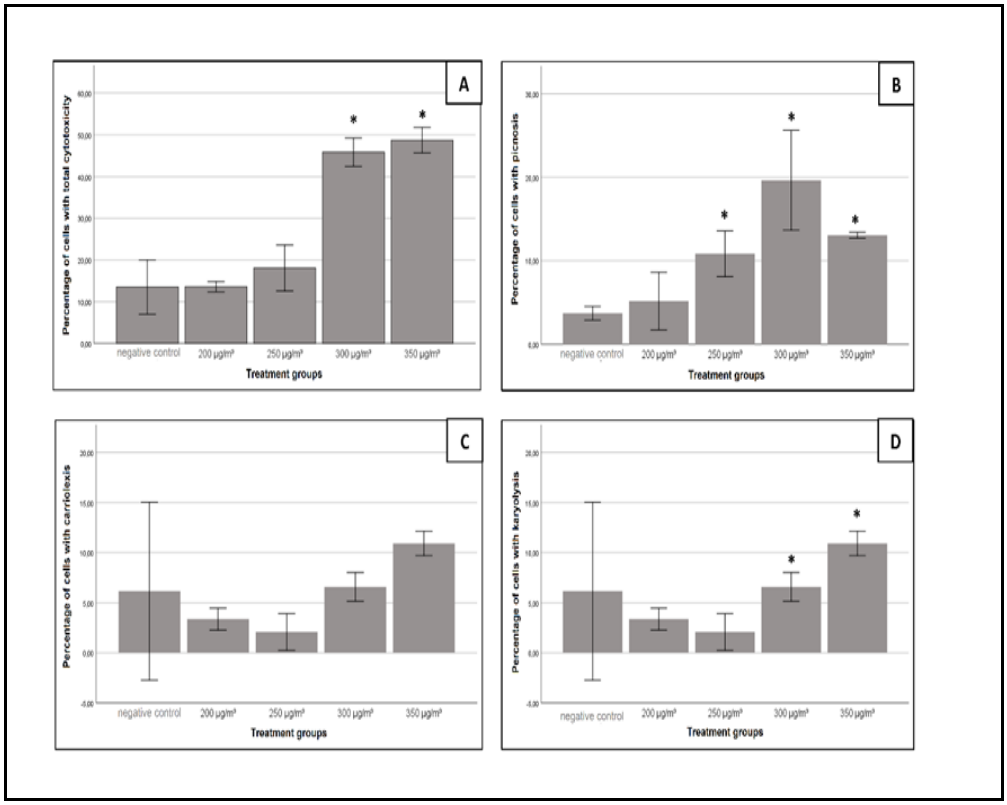

Figure 3: Effect of Sulfur Dioxide (SO2) on the Variation of the Percentage of Cells with Cytotoxicity According to the Treatment Groups. (A) Percentage of Cells with Total Cytotoxicity. (B) Percentage of Cells with Picnosis. (C) Percentage of Cells with Carriolexis. (D) Percentage of Cells with Karyolysis. The Bars Represent Mean ± DS. The Significant Differences (P <0.05) Respect to the Control Group are Represented by an Asterisk.

CONCLUSIONS

It was shown that in concentrations up to 300 µg/m3 of SO2 in air it is capable inducing toxic effects on the proliferation of buccal epithelial cells generating the appearance of micronuclei, genotoxic damage, and nuclear aberrations such as picnosis and karyolysis, or cytotoxic damage, which it may be related bye the excess of ROS generated by SO2 in the body while genotoxic damage is generated from 350 µg/m3.

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