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Genotoxic and cytotoxic effect of exposure to thallium on mature spermatozoa of mice

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Efecto genotóxico y citotóxico de la exposición al talio en espermatozoides maduros de ratones

Resumen

Introducción. Los efectos adversos de los químicos en la reproducción masculina es un área de preocupación creciente, actualmente el Tl es utilizado principalmente en la fabricación de dispositivos electrónicos, interruptores y cierres; así como en plantas generadoras de energía, fábricas de cemento y fundiciones. Objetivo. Evaluar la calidad espermática y el daño genético de los espermatozoides en ratones expuestos oralmente a Tl. **Materiales y métodos**. Se utilizaron ratones macho y dosis de Tl de 5, 15 y 25 mg/kg/5d, se evaluaron los parámetros de calidad espermática y el daño al ADN por la técnica de SCSA. **Resultados.** La exposición a Tl afecto la motilidad, la viabilidad y la morfología espermática. Se observaron alteraciones en la estructura del ADN (%DFI y %HSD) de los espermatozoides. El Tl se podría considerar reprotóxico, ya que altera capacidad reproductiva.

Palabras clave: Talio; Calidad Espermática; SCSA

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Abstract

Introduction. The adverse effects of chemicals on male reproduction is an area of growing concern. Currently, Thallium (Tl) is used mainly in the manufacture of electronic devices, switches and closures, as well as in power plants, cement factories and foundries. Aim. To evaluate sperm quality and genetic damage of sperm in mice exposed orally to Tl. Materials and methods. Male mice were treated with Tl doses of 5, 15 and 25 mg/kg/5d, sperm quality parameters and DNA damage were evaluated by the Sperm Chromatin Structure Assay (SCSA) technique. Results. Exposure to Tl affected sperm motility, viability and morphology. Alterations in the DNA structure (%DFI and %HSD) of the spermatozoa were observed. Tl could be considered reprotoxic, since it alters reproductive capacity.

Keywords: Thallium; Sperm Quality; SCSA

INTRODUCTION

The use of metals is of great relevance today, since they are used in the manufacturing processes (Martin-Hernandez, 2004; Coello & Burgos, 2012). For example, thTallium is used as an anticorrosive, in the chemical industry, in the production of cadmium and the refining of olefins, in optics to increase refraction, in imitation jewelry and fireworks, in the manufacture of crystals, jewelry, electrical equipment and electronics, semiconductors, optical systems, infrared spectrophotometers (bromine crystals and thallium iodide), thermometers to measure low temperatures, scintillation counters and fiberglass cables for telecommunications, among others. It has also been used for criminal purposes due to its lack of color, smell and taste (Goyer, 2001; Aristizabal and Zuluaga, 2012).

Tl has no biological function, on the contrary, it is considered a cumulative poison (The half-life of Tl is 3.08 days) capable of producing harmful effects on health (Rodríguez-Mercado and Altamirano-Lozano., 2013). Tl crosses biological membranes, is distributed by the systemic circulation throughout the body and is excreted through body fluids, hair, nails, feces (26.4%) and urine (51.4%). A portion of Tl accumulates in organs and tissues, and gradually returns to the bloodstream (Rodríguez-Mercado and Altamirano-Lozano, 2013).

Until the early years of the 20th century, Tl salts were widely used to treat diseases such as syphilis (since 1883), to reduce night sweats in tuberculosis patients (since 1898), for hair removal (since 1897), to treat ringworm on the scalp (until 1919) and to kill rodents (since 1920) (Montoya-Cabrera et al., 1991). Tl salts are odourless, tasteless and extremely toxic; these aspects led to Tl compounds being chosen for poisoning homicides during the early years of the 20th century (Korenman, 1963). After the federal government of the United States banned Tl as a rodenticide in 1972, it ceased to be used in most industrialized countries where mainly pesticides contained this metal (Beliles, 1994).

Tl is one of the main pollutants, along with lead (Pb), cadmium (Cd) and mercury (Hg), on the list of the United States Environmental Protection Agency (ATSDR, 1992).

The severity and damage caused by exposure to Tl produces one of the most severe intoxications known to humans, involving different organs and tissues. The clinical picture of intoxication by this metal depends on the time, level of exposure, range of absorption, in particular individual susceptibility (ATSDR, 1992). Acute exposure can affect the central and peripheral nervous systems, while chronic exposure results in brain, spinal cord, and peripheral nerve involvement (Moore et al., 1993).

The main clinical manifestations of acute Tl intoxication consist of dermatological features such as alopecia, hyperkeratosis and the presence of Mee's lines in the nails (Repetto et al., 1998). Neurological symptoms include dysesthesia, neuropathic pain, muscle weakness, cranial nerve palsies, tremor, seizures, coma, and death (Galván-Arzate and Santamaría, 1998). The term encephalopathy implies a variety of conditions, such as loss of control of the person and memory impairment, decreased intellectual capacity with irreversible dementia, and hallucinations (Davis et al., 1981).

Exposure to high levels of heavy metals such as Pb, Cd, Hg, and Tl contribute to infertility problems in men (Benoff et al., 2000). On the other hand, little has been explored at the male reproductive level on the effects of Tl. It has been reported in the ATSDR, (1992) that the reproductive organs of animals, especially the testicles, show damage after drinking water contaminated with small amounts of Tl for 2 months.

In study the germ cell loss was observed in an *in vitro* testicular cell culture study with a significant reduction in prepachytene and pachytene stage spermatocytes and changes in sertoli cell shape (Gregotti et al., 1992). In experimental animals, a toxicity similar to that mentioned for humans and other conditions is manifested. In rats exposed to an acute dose of 20 mg/kg TI nitrate, they experienced difficulty breathing and death (EPA, 2009). On the other hand, subchronic oral treatment (15 weeks) of 0 to 3.9 mg/kg of thallium acetate caused alopecia, kidney damage and death in some animals, in addition to acute subcutaneous injection of 20 and 50 mg/kg of the same gene compound-induced inflammation of the small intestine, colitis, as well as gradual degeneration in kidney, liver, and nervous tissue (EPA, 2009).

Toxicity studies in rat and chicken embryos provide evidence that exposure to Tl during development can produce abnormalities (including effects on the nervous, vascular, and skeletal systems), reduced body weight in fetuses. In male mice, oral doses of 0 to 3

mg/kg Tl per day for a period of 6 months were found to decrease motility and cause sperm deformation (Wei, 1987). Recent *In vitro* studies have shown that Tl compounds are capable of interacting with deoxyribonucleic acid (DNA) and inducing cytotoxicity (Rodríguez-Mercado and Altamirano-Lozano, 2013).

For all of the above, the objective of this work was to evaluate the sperm quality and the genetic damage of the sperm in mice exposed orally to Tl. In this study, doses of 5, 15 and 25 mg/kg/5d of Tl were used, these concentrations are below the LD50 for mice (31.02 mg/kg) (http://en.cnki.com.cn/ Article en/CJFDTOTAL-GWYZ201103013.htm).

MATERIALS AND METHODS

Reagents

Technical grade thallium acetate (Tl_2SO_4; 55.2% purity) and $NaCl_2$, Trypan Blue was purchased from Sigma Aldrich Company (St. Louis, MO). Ethanol, Triton x-100 was obtained from J.T. Baker Mallinckrodt (Mexico). Acridine orange and SARH-FITC was from Amersham (Amersham, UK), EDTA was from BioRad (USA) and Tris Ultra-Pure was from Invitrogen (New Zealand), Papanicolau OG-6 stain from Merck (Darmstadt, Germany). All other reagents were chemicals of the highest quality.

Animals and treatments

The protocols were approved by the animal ethics committee of the Autonomous University of Guerrero, Mexico. ICR-CD1 male mice (8 weeks old) were purchased from the animal facility of the Institute of Biotechnology of the National Autonomous University of Mexico, Cuernavaca, Mexico. All animals were acclimatized for two weeks before starting the experiments. Mice (10 weeks of age) were randomly assigned into control and treated groups and placed in individual boxes, maintained on 12-h light-dark cycles, with food and water *ad libitum*. Tl_2SO_4 was dissolved in drinking water and administered orally at doses of 5, 15 or 25 mg/kg/orally (p.o.)/d/5 days. Controls received only vehicle (50 µL of water). Six animals were administered with Tl_2SO_4 and three were administered only with water (control group) by dose and exposure time.

Sperm isolation and analysis

Mice were euthanized 24 hours (h) after the last administration, corresponding to cells that were in the epididymal maturation stage or elongated spermatid (Peirce and Breed, 2001), at the time of administration. The tail of the epididymis and vas deferens were

removed, cleaned of fat and blood vessels and connective tissue, the spermatozoa were washed with saline solution. Sperm cells were analyzed by light microscopy according to WHO, (2010), including sperm concentration by hemocytometer method, percentage of viable cells using 0.5% trypan blue, progressively motile cells by phase contrast microscopy (200 cells were counted) and sperm morphology, followed by the modified Papanicolao technique and its classification described by Wyrobek et al., 1983).

Evaluation of DNA integrity and sperm chromatin structure by flow cytometry (SCSA).

The Sperm Chromatin Structure Assay (SCSA) technique, modified by Evenson (2016), was used. The SCSA technique evaluates the susceptibility of sperm chromatin to acid denaturation in situ. The samples were incubated with acridine orange fluorochrome (NA), which has metachromatic properties that allow it to intercalate between the bases of double-stranded DNA and emit a green fluorescence (non-denatured DNA) and when it intercalates with double-stranded DNA simple emits a red fluorescence (denatured DNA). DNA Fragmentation Index (%DFI) and High DNA Fluorescence (%HDS), which represent DNA damage and the percentage of cells with immature chromatin, were evaluated. For this, an aliquot of 2×10^6 spermatozoa was resuspended in 200 µL of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 0.01 M EDTA, pH 7.4), and sonicated (Ultrasonic processor GE-130) at a maximum 60% power at 4 ºC/3 min to remove contaminating somatic cells. Subsequently, to an aliquot of 100 µL of this suspension (1×10^6 sperm), 200 µL of the permeabilizing solution (0.08 N HCl, 0.15 M NaCl and 0.1 % Triton X-100, pH 1.2) were added and the sample was left rest on a bed of ice for 30 sec to facilitate the entry of the fluorochrome into the cells. Immediately afterwards, 600 µL of the staining solution (0.1 M citric acid, 0.2 M Na_2HPO_4 $7H_2O$, 0.001 M EDTA, 0.15 M NaCl and 600 µL NA) were added and the sample was left to stand on ice for 3 min. Subsequently, the cell preparation was deposited in a polypropylene tube for analysis in a flow cytometer (FACSort Becton Dickinson, CA). A reference sample was evaluated in each run as a quality control. Data were analyzed using the SCSASoft® program (SCSA Diagnostic, Inc., Brookings, SD).

Statistical analysis

Data from two independent experiments are presented as mean ± SD and used for statistical analyses. Comparisons between treated and control groups were made by analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. The variables were correlated by means of Pearson's parametric analysis that was used for the comparison of the control groups of the two experiments. All data were analyzed using STATA version 17.0 statistical software (Stata Corp., College Station, TX). For all comparisons, statistical significance was assigned at $p<0.05$.

RESULTS

Thallium effect on body weight and relative weight of important organs.

In mice treated with Tl at doses of 5, 15 and 25 mg/Kg/5d, a decrease in body weight was observed in all doses, however, the dose of 25 mg/Kg/5d showed a remarkable significant difference compared at the other doses and presented a greater effect with respect to the control group. Regarding the organs of metabolic importance, the liver had a decrease in relative weight of 0.3 times in the treatment with the different doses, the lung, seminal vesicles and coagulating glands decreased 0.3 times and the spleen decreased 0.06 times in the highest dose. On the other hand, at the dose of 15 mg/kg/5d/po, the kidney showed a significant increase of 0.3 times, compared to the control group (Table 1).

Table 1. Effect of thallium exposure on body and relative organ weight at doses of 5, 15 and 25 mg/kg/5 days/v.o.

Grup	Body weight (BW)	Liver	Kidney	Testis	Lung	Spleen	Seminal vesicles and coagulating glands
$C_2H_3O_2Tl$ (mg/kg/d/5d/ v.o)							
Control	43.6±3.8	2.3±0.2	0.6±0.1	0.2±0.1	0.3±0.1	0.16±0.04	0.3±0.06
5 mg/kg	40.5±2.9	2.01±0.3	0.6±0.07	0.2±0.06	0.3±0.09	0.1±0.01	0.2±0.03
15 mg/kg	42.4±5.4	2.0±0.5	0.8±0.1*	0.2±0.04	0.3±0.01	0.1±0.05	0.2±0.08
25 mg/kg	38.5±6.3*	2.0±0.4*	0.7±0.1	0.2±0.09	0.2±0.08*	0.10±0.03*	0.2±0.05*

Data are expressed as mean ± SD (n=4 control; n=6 treated animals per dose) of two independent experiments. ANOVA tests and the Bonferroni multiple comparison test were used. *Significant difference ($p<0.05$).

Thallium effect on sperm quality.

Exposure to Tl altered sperm quality parameters at the different doses administered (5-, 15- or 25-mg/kg/5d/v.o.). Viability decreased by 32%, 38% and 45% at doses of 5-, 15- or 25-mg/kg/5d/v.o, respectively (Fig1a). Sperm motility also decreased by 37%, 42%, and 57% at the 5-, 15-, or 25-mg/kg/d/5d/ dose, respectively, compared with the control group (Fig. 1b).

Regarding the morphology of the spermatozoa, this has a decrease of 3% only at the highest dose (Figure 1c); the morphological alterations observed were mainly in the head of the spermatozoon (head in the shape of a carnation and in the shape of a pin) everything was compared with respect to the control group.

Effect of thallium on sperm DNA damage

The %DFI parameters showed a significant decrease of 0.46, 0.52 and 1-time, this in the dose of 5-, 15- or 25-mg/kg/5d/v.o. respectively, (Figure 2a). On the other hand, in the %HSD parameter which evaluates the degree of chromatin condensation, a significant decrease of 1.09, 0.61 and 0.53-fold was observed at the dose of 5-, 15- or 25-mg/kg/ 5d/o.v., compared to the control group (*P<0.05) (Figure 2b).

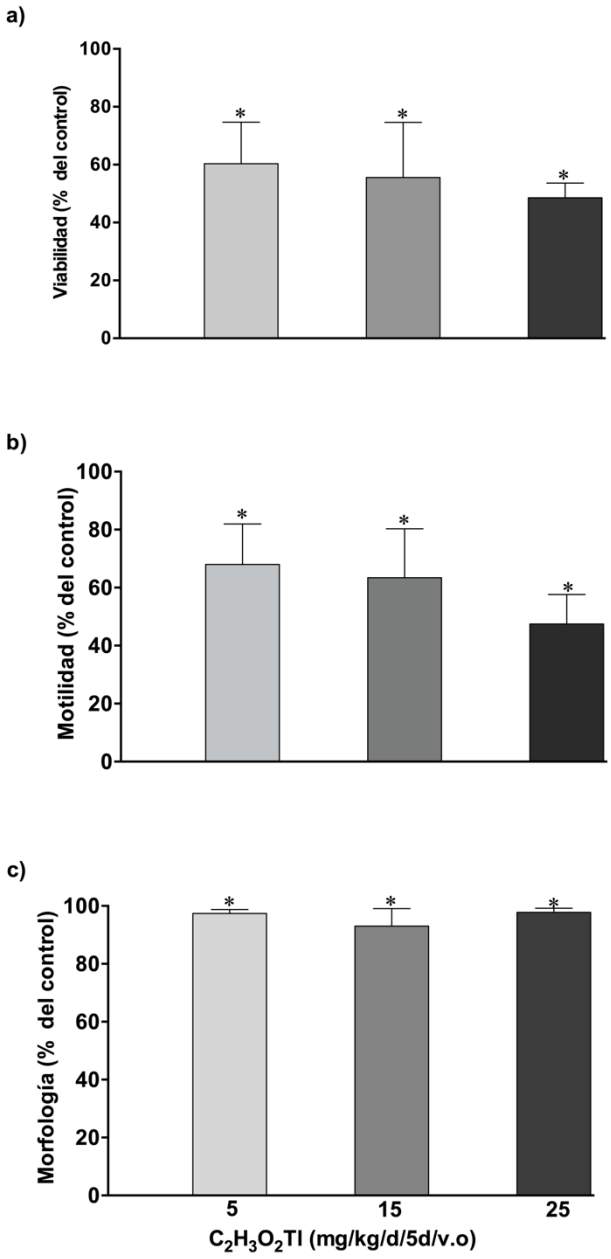

Figure 1. Effects of Tl exposure on sperm quality. The bars represent the mean ± SD. *Statistically significant difference with respect to the control group P<0.05 Vs the control group (ANOVA and Bonferroni multiple comparison test (n = 4) Vs the treated group (n = 6) in mature spermatozoa.

Figure 2. DNA damage and chromatin compaction caused by exposure to Tl. a) % DNA (DNA fragmentation index) and b) % HSD (Degree of chromatin condensation). The bars represent the mean ± SD. *P<0.05 vs control group (ANOVA and Bonferroni multiple comparison test. (n = 4) vs treated group (n = 6).

DISCUSSION

Humans have been in contact with metals since the beginning of humanity and they have played a considering role in the development human civilization. However, some metals are considered contaminants, because of the burning of fossil fuels, intense industrial activity, as well as various natural processes (volcanic eruptions), have increased their dispersion and concentration, which has induced negative effects on the environment

and health (Moreno-Sánchez, and Devars, 1990; Maluszynski, 2009). Studies in humans and animals indicate that Tl compounds are readily absorbed through various routes of exposure, but few studies provide quantitative measures of absorption (ATSDR 1992).

Mulkey and Oehme, (1993) reported that water-soluble thallium salts are rapidly and completely absorbed from the respiratory, gastrointestinal (GI), or skin tracts. Although the mechanisms of Tl toxicity are not exact and little is known, since Tl interacts with cells at different levels. Tl is capable of mimicking the potassium ion in most biological cases, due to the same ionic radius and to the fact that the cell membrane is unable to differentiate between these two cations. Tl follows potassium distribution and thus disrupts many potassium-dependent processes, for example, Tl can substitute for potassium Na+/K+-ATPase (Britten and Blank, 1968).

It has been shown that Tl associates with the Na/K-ATPase channel with an affinity 10 times greater than potassium. Once there are intracellular accumulations of Tl, it interferes with the function of a series of enzymes through the binding of hydrogen sulfide and, finally, the inhibition of cellular respiration, altering calcium homeostasis and the interaction with riboflavin and cofactors derived from riboflavin (Mulkey and Oehme, 1993), this characteristic in our system possibly allows Tl to be easily absorbed by the cells of the peritoneum, reach the bloodstream, distribute to the bone marrow and other tissues, as observed when administering thallium acetate and other metallic compounds via i. p. and oral in different murine models (EPA, 2009).

Another possible mechanism of toxicity is the ability of Tl to react with thiol groups (WHO, 1996). Tl affects the metabolism of glutathione, a non-protein thiol whose main function is to maintain plasma oxidant homeostasis by defending it from oxygen species. Furthermore, glutathione plays a crucial role in heavy metal toxicity through its SH group, which ends up in excretion. The deterioration of this protection system can result in the accumulation of reactive oxygen species, which could negatively affect different molecules and their related cellular processes (Brailovskaya., 2001).

Hanzel, et. al., (2005) investigated the effects of thallium hydroxide on glutathione metabolism in vitro using rat brain cytosolic fractions. A decrease in Tl hydroxide, inhibited glutathione peroxidase and glutathione reductase (Halliwell and Gutteridge., 1999) were observed. The levels of thallium acetate administered are in a range where a

biologically relevant dose is manifested because the LD50 for mice is 37 mg/kg i.p or 35 mg/kg orally; In addition, a study was conducted in compliance with the mandates of good laboratory practices (GLP) of the EPA where mice exposed to concentrations of 0.01, 0.05 or 0.25 mg/kg/day of an aqueous solution of thallium sulfate were used. for 90 days to which the results show, it was observed that the mice were able to withstand these doses (Repetto and del peso, 2012; EPA, 2009). It has also been reported in the ATSDR, (1992) that the reproductive organs of animals, especially the testicles, show damage after drinking water contaminated with small amounts of Tl for 2 months, these results agree with what was reported by Galván-Arzate and Santamaría, 1998; EPA, 2009, where it was observed that the kidneys, fatty tissue, the lung, the brain and the gonads tend to accumulate this metal, since it can cross the blood-brain and blood-testicular barriers. Therefore, our results are related to those already reported, where body weight decreased only at the highest dose, in addition to a decrease in the weight of organs of metabolic interest, such as liver, lung, spleen, seminal vesicles and coagulation glands, this study showed that the 15 mg/kg dose affected only the kidney, increasing its weight.

Animal studies have revealed that, once they have entered the cell, some metal ions bind to a specific protein, metallothionein, this low molecular weight protein is present in cells of the liver, kidney and other organs and tissues their sulfhydryl groups can bind six ions per molecule. A greater presence of metal ions induces the biosynthesis of this protein. Metallothionein also serves to maintain homeostasis of vital copper and zinc ions because it can bind zinc, copper, cadmium, mercury, bismuth, gold, cobalt, and other cations (WHO, 1996).

Male reproductive function has been significantly affected during the last 50 years and this alteration could be related to exposure to different toxins and environmental contaminants (Carlsen et al., 1992). Although studies on sperm quality with Tl are scarce, it has been reported that in male mice at oral doses of 0 to 3 mg/kg of TI per day over a period of 6 months, decreased mobility and caused sperm deformation (Wei, 1987). Regarding our study, alterations in sperm quality were observed, which were manifested in the following order: viability > motility > morphology. These results agree with those reported by Wei (1987), where he observed some of the testicular effects such as: tubular epithelial imbalance, cytoplasmic vacuolization, and reduced sperm motility

The distension of the smooth endoplasmic reticulum in Sertoli cells is due to the reduction in the activity of β-glucuronidase, as well as to the study carried out by Gregotti et al., (1992), who found alterations in the activity of β- glucuronidase and ultrastructural changes in Sertoli cells after 60 days of treatment altering sperm quality. It is currently unknown that Tl can directly interact with and damage nuclear DNA, although some Tl compounds have a strong affinity, particularly for the nitrogenous base guanine (Howerton, Sines, VanDerveer & Williams, 2001). It has been proposed that DNA damage can be caused by free radicals, which are generated by endogenous sources, such as normal physiological processes within which respiration is found, or by exogenous sources, such as interactions with xenobiotics such as metals (Oliveira et al, 2009). When the antioxidant control mechanism is overcome by reactive species, the redox potential of the cell changes to a state of stress, increasing the potential for damage to biomolecules such as DNA, lipids and proteins (Rodríguez-Mercado and Altamirano-Lozano, 2006). There are studies showing extreme oxidative damage due to the presence of a highly reactive product of the sulfhydryl group in the active site (Cys-283) that oxidizes, modifying the high affinity of Tl with the Thiolate group (-SH), giving rise to to enzyme inhibition (Korotkov, 2021). There is little information on the damage that Tl generates in sperm DNA, so its mechanism is possibly the same as that of cadmium, arsenic and vanadium, they are genotoxic, because they can induce single and double-stranded DNA breaks (Rodríguez-Mercado and Altamirano-Lozano., 2013).

Oliveira et al, (2009) observed that cadmium destabilizes the structure of sperm chromatin in mice and suggested that this effect occurs during the post-meiosis phase of spermiogenesis, where the transition from histones to protamines occurs. Due to its great chemical similarity with phosphate, vanadium can interfere with several enzymatic systems such as Na^{+}-K^{+}-ATPase, H^{+}-K^{+}-ATPase and Ca^{2+}-Mg^{+}-ATPase, which are inhibited in the presence of this metal (Nechay., 1984). Vanadium also inhibits enzymes involved in the formation and hydrolysis of ATP, such as adenylate kinase and ATP phosphohydrolase (Nechay., 1984).

It can also interfere with metabolic processes such as glycolysis by inhibiting the enzymes glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate. Other enzymes that are inhibited by vanadium are ribonucleases and DNA polymerases, so the metal

causes damage at the level of DNA synthesis (Nechay, 1984; Kikkert, 2010). During spermatogenesis, sperm DNA is initially arranged in the form of nucleosomes, being linked, like the DNA of somatic cells, to histones, which will later be replaced almost entirely by protamines, which are responsible for give the characteristic compaction to the sperm nucleus (Oliva et al., 2006). When this substitution of histones for protamines fails, excessive histone persistence is generated, resulting in immature sperm chromatin (Oliva et al., 2006).

There is evidence of how Tl affects organisms, however, little is known about the way it induces genetic damage, this is due to the fact that the data available to date are not clear or conclusive (EPA, 2009; Repetto and Del weight, 2012). A study carried out by Zasukhina, (1983), using thallium carbonate, it was observed that it induces DNA breaks in embryonic cells of C57BL/6 mice and CBA rats. On the other hand, Felipe-Reyes et al., 2011 carried out a study where an in vitro model of human peripheral blood cells was used, they found that thallium acetate, as in other presentations (thallium sulfate, thallium chloride) increases the frequency of cells with ACE (including and excluding gaps). Tl can be considered as a reprotoxic compound since it alters reproductive capacity, inducing genotoxicity and alterations in sperm quality in this study.

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