

Teratogenic effect on bone tissue development in *Rattus norvegicus* Wistar strain, induced by the presence of arsenic

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Abstract

In this study we evaluated the possible teratogenic effects of arsenic (As) in the same concentrations recorded in water Zimapan, Hidalgo, using specimens of *Rattus norvegicus* Wistar strain, pregnant. Were subjected to varying concentrations of sodium arsenate (1887.9 mg/L, 943.9 mg/L and 0.5 mg/L), concentration, 0.5 mg/L corresponds to the registered Zimapan water, Hidalgo and chromium trioxide (166.0 mg/L) used as positive control and 5% sucrose as a negative control. The specimens were exposed from day 7 through 15 of gestation, and were sacrificed on day 19 of gestation by inhalation of chloroform. We analyzed reproductive harm the mother, as well as alterations and lack of ossification of the bone tissue of the fetus. The highest dose tested (1887.9 mg/L) produced more reproductive harm in mother resorptions caused by preventing the continuation of embryonic development. Concentrations of 0.5 mg/L to 943.9 mg/L, resulted in lack of ossification ribs, sternbrae, metacarpals, phalanges previous metatarsals and phalanges posterior sacral vertebrae.

Keywords: sodium arsenate; teratogenic; reproductive harm; lack of ossification; *Rattus norvegicus*.

Efecto teratogénico sobre el desarrollo del tejido óseo en la cepa Wistar de *Rattus norvegicus*, inducida por la presencia de arsénico

Resumen

En este estudio evaluamos los posibles efectos teratogénicos del arsénico (As) en las mismas concentraciones registradas en el agua de Zimapan, Hidalgo, usando especímenes de la cepa Wist de *Rattus norvegicus*, preñada. Se sometieron a concentraciones variables de arseniato de sodio (1887.9 mg/L, 943.9 mg/L y 0.5 mg/L), concentración, 0.5 mg/L corresponde al agua registrada de Zimapan, Hidalgo y trióxido de cromo (166.0 mg/L) utilizados como control positivo y 5% de sacarosa como control negativo. Las muestras se expusieron del día 7 al 15 de gestación y se sacrificaron el día 19 de gestación por inhalación de cloroformo. Analizamos el daño reproductivo de la madre, así como las alteraciones y la falta de osificación del tejido óseo del feto. La dosis más alta probada (1887.9 mg/L) produjo más daño reproductivo en las resorciones de la madre causado por la prevención de la continuación del desarrollo embrionario. Concentraciones de 0.5 mg/L a 943.9 mg/L, resultaron en la falta de costillas de osificación, esternbras, metacarpianos, falanges metatarsales previos y falanges de vértebras sacras posteriores.

Palabras clave: arseniato de sodio; teratogénico; daño reproductivo; falta de osificación; *Rattus norvegicus*.

1. Introduction

Pollution in natural water, both chemical contaminants (polychlorinated biphenyls (PCBs), heavy metals, dioxins, chemicals, etc) such as biological, is a worldwide problem. Actually, few populated areas in developed and developing

countries, which are not subject to contamination [1].

The accumulation of heavy metals in the environment and in biological systems, either by adsorption, precipitation and other forms of natural association, including bioaccumulation, are one way to minimize transport and spreading. However, this can have negative consequences for the ecological environment due

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to leaching of chemical species in significantly higher amounts, are accessible to aqueous systems. Consequently man can be incorporated by the ingestion of water and/or through food chains [2].

Arsenic (As) is not a metal, however it has properties of heavy metals, their toxic effect is similar to that of Mercury (Hg) and Lead (Pb). Forms As (III) or arsenite, are the most toxic [3]. Arsenic (As) is highly distributed in nature and is present in many work activities. With the growth of industrial activities, the sources of environmental contamination with this element and other heavy metals have increased significantly [4]. The leaching of mine tailings from gold and other minerals left in previous decades and centuries, are a significant source of As contamination in groundwater systems, such as the State of Hidalgo, which has a long tradition of mining activity [1].

The arsenic found in the oceans is very low values, close to 0.001 - 0.008 mg/L. Their concentration in the rivers is highly variable and has been identified from 0.1 mg/L to 1 mg/L [5]. As the average content in drinking water is approximately 2.4 mg/L. Arsenic in water, acid commonly found as As (V), H₃AsO₄ or deprotonated form, which is a less toxic form. Also, is the ace in the form of As (V) (as H₂AsO₄ and [HAsO₄]-₂) a less toxic As (III) [6].

It has been reported that the ingestion of small amounts of As, at a prolonged level, produces affectations to the bone marrow and the placenta and are stored in the bone tissue. Also has been associated with various processes carcinogenic and teratogenic in several bioassays [7]. In some regions has been identified endemic regional chronic hydroarsenism, reported at concentrations of 0.21 mg/L to 12.6 mg/L. Concentrations sufficient to cause long-term poisoning in the human population [5].

They have identified a large number of natural areas with groundwater, which present As content exceeding 50 µg/L in different parts of the world (Fig. 1). The major problems cited in the literature are located in Argentina, Bangladesh, Nepal, Chile, China, Hungary, India (West Bengal), Mexico (Laguna Region and Zimapán Valley), Romania, Taiwan, Vietnam and the U.S., being in latter and in Bangladesh

where they have been the subject of further study. In addition there are other areas directly related to hydrothermal processes in Argentina, Chile, Japan, New Zealand, USA, Iceland, France, Dominican Republic and Kamchatka. Areas with arsenic contamination problems related to mining and mineral deposits have been recognized in many parts of the world, being the most outstanding cases of Ghana, Greece, Thailand, Chile and the U.S. [8].

In Hidalgo, the State Office of the National Water Commission is aware of the presence of high levels of As in the water is supplied by wells Zimapán City (Fig. 2).

A study on the concentrations of this element during the period March 1992 to March 1993, confirmed the presence of As in these wells [6]. Recorded concentrations exceeded the maximum permissible limits for 2005, which is 0.025 mg/L, according to Mexican legislation in 2000 [9]. Also, there were several monitoring of As to 1998, concluding with the closing of Well No. 4 (the Muhi) presented a concentration of 1.5 mg/L of As. The results of the analysis of Zimapán water samples (September 2002) show that the content of As in concentrations ranging from 0.30 mg/L to 0.50 mg/L, 12 to 20 times more than what is established by the NOM -127-SSA1-1994 [2,9-10]. The World Health Organization (WHO) has concluded that 1/10,000 population at risk of getting skin cancer caused by the daily intake of water with As at concentrations of 0.002 mg As/L. Therefore, the maximum As for water intended for human consumption in some European Union countries such as Spain, has been reduced to values of 0.010 mg/L [6].

It is essential to evaluate the causes and effects caused by environmental pollution. This leads to environmental monitoring is absolutely necessary to identify risks on human health and the ecosystem [11]. The monitoring of environmental pollution can be done primarily through chemical analysis. However, it should be reinforced through a biological assessment of toxicology, assessing the effects of xenobiotics on ecosystems and individuals using bioindicators or biomonitors, which together provide a comprehensive assessment of the environment and biological systems [12].



Figure 1. Global distribution of aquifers with high contents of As
Source: taken from [8].

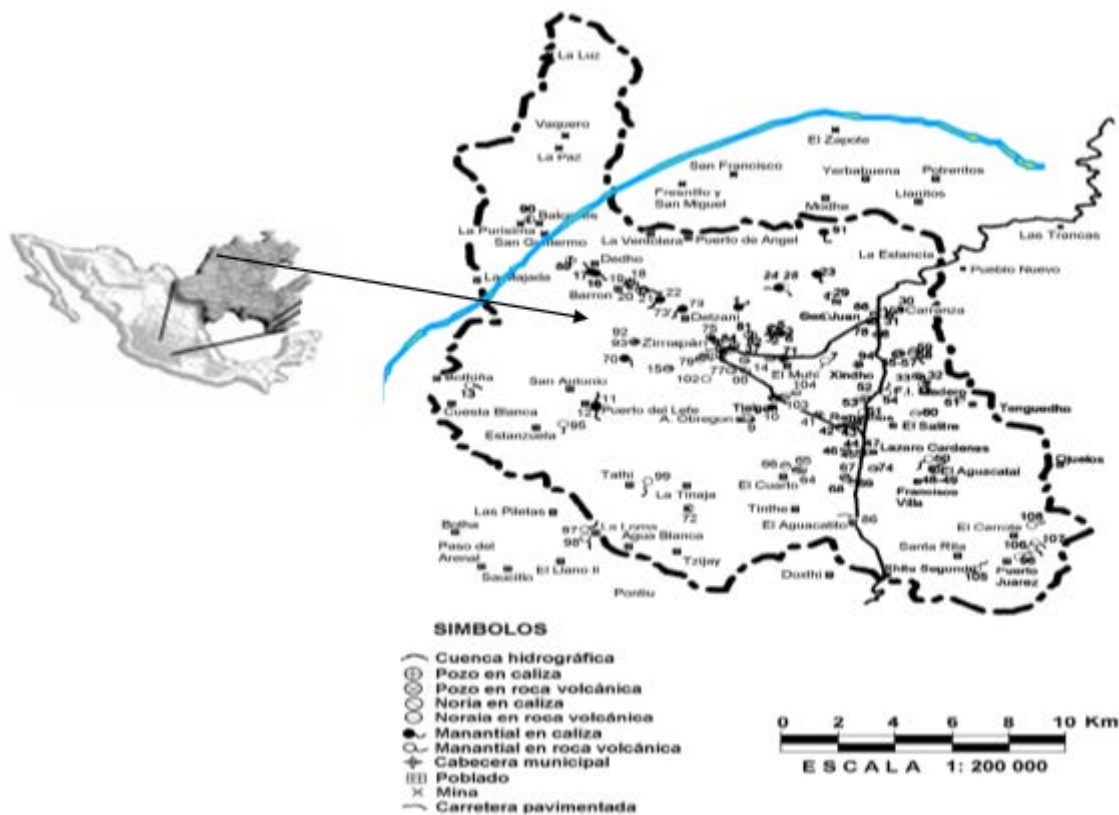


Figure 2. Zimapán City, Hidalgo, belongs to RH-26 hydrological Panuco northern part of the Basin of Mexico, Sub-basin of Rio Moctezuma. Blue line border area in the northern part of the Basin of Mexico
Source: taken from [10].

Bioassays are a type of biomarker that may be from viruses to human tissue culture, which under controlled conditions (in vivo and in vitro) allowed us to correlate the effect of the agent and/or physical chemical biological level [13]. There are different levels where you can express the hurt biological level, these can be, morphological, physiological and/or behavioral. Of these, the mutagenic, carcinogenic and teratogenic effects are important, because of their frequency and effects that impact on social, economic and health [14].

A lot of bioassays were used to assess environmental quality, one of the most used is that of *Rattus norvegicus*. This bioassay has many advantages for use in the laboratory, can evaluate the genotoxic effect and/or teratogenic induced by various pollutants. Furthermore, it is a mammal, allowing to correlate the results between rat and man [15].

The technique that is apparent in fetuses can observe the skeletal system, as it is in other organisms, because no dislocation of the same. One advantage of this technique is that organisms are preserved for a long time, allowing occasions carefully review them as necessary. This technique is used in rats to observe skeletal malformations to identify ossification centers thereof and/or delayed development, among others, naturally or induced by exposure to xenobiotic agent [16-17].

2. Methodology

2.1. Establishment of experimental plots

We used 50 individuals (pregnant females) of *R. norvegicus* who were on their first day of gestation. They were randomly divided into five treatment groups with 10 specimens each:

- Lot 1: 5% Sucrose, concentration corresponding to a negative control competitor.
- Lot 2: 166.00 mg/L of chromium trioxide (CrO_3) [18], concentration corresponding to a concurrent positive control.
- Lot 3: 0.5 mg/L of sodium arsenate (Na_2HAsO_4) corresponding concentration Zimapán waters, as it is of interest for this work to know their teratogenic effects on the skeletal system of *R. norvegicus*.
- Lot 4: 943.9 mg/L of Na_2HAsO_4 , concentration corresponding to the quarter of the CL_{15} subtoxic concentration.
- Lot 5: 1887.9 mg/L Na_2HAsO_4 , concentration corresponding to half the CL_{15} subtoxic concentration.

The administration of the compound to all experimental plots was performed by mouth with chronic exposure. The administration was performed during organogenesis of *R. norvegicus*, from the 7th to 15th day of gestation. The specimens were examined daily for signs of toxicity notorious,

Table 1.
Dawson made transparent technique

Skeletal abnormalities	Control		Experimental treatments		
	Sucrose, %	CrO ₃ , mg/L	Na ₂ HAsO ₄ , mg/L		
	5	166	0.5	943.9	1887.9
Total analyzed fetuses	85	85	85	85	85
*Ribs					
N° Supernumerical	0	1/1.17	0	2/2.35	2/2.35
*Ribs					
Lack ossification, N°/%	0	43/50.58*	21/24.70*	17/20.00*	5/5.88*
*Estenebras					
N° Merged	0	0	0	0	0
*Estenebras					
Lack ossification, N°/%	0	70/82.35*	49/57.64*	32/37.64*	50/8.82*
*Pastern					
N° Absence	0	0	0	0	0
*Pastern					
Lack ossification, N°/%	0	40/7.05*	30/35.29*	32/37.64*	8/9.41*
* Previous phalanges					
N° Absence	0	0	0	0	0
* Previous phalanges					
Lack ossification, N°/%	0	66/77.64*	56/65.88*	65/76.47%	48/56.47
*Hocks					
N° Absence	0	0	0	0	0
*Hocks					
Lack ossification, N°/%	0	40.47*	31/36.47	32/37.64	8/9.41
* Phalanges later					
N° Absence	0	0	0	0	0
* Phalanges later					
Lack ossification, N°/%	0	76/89.41*	75/88.23*	69/81.17*	66/77.64*
* Sacral vertebrae					
N° Absence	0	0	0	0	0
* Sacral vertebrae					
Lack ossification, N°/%	1/1.17	71/83.52*	76/89.49*	70/82.35*	69/81.17*

* Significant difference from control group, Dunnett's test, p<0.05 [23-24].

Source: Own elaboration, modified by Stapler [19]

impending abortion or premature delivery. In the last two cases, the specimens were sacrificed before the 19th day of gestation.

2.2. Sacrifice

The animals of all lots were sacrificed on gestation day 19th by inhalation of chloroform [19].

2.3. Hysterectomy and recording in the rat reproductive harm progenitor

After sacrifice of copies, a hysterectomy is practical to each individual pregnant, assessing reproductive damage by assessing the number of fetuses, number of implantations, early and late resorptions. Independently, fetuses were removed, recording the number of them and placing them in 96% alcohol for later evaluation teratogenic damage these.

Made transparent fetuses: To assess teratogenic damage through skeletal abnormalities and lack of ossification of the bone tissue, the analysis was performed according to the technique bone Dawson Staples modified in order to detect damage induced by the treatment (Table 1) [19].

Analysis and recording of changes in bone tissue in fetuses: For the record of the present skeletal changes were taken into account the number and condition of ribs, sternebrae, metacarpals, phalanges, metatarsals and sacral vertebrae because they are the main centers of ossification (Fig. 3) recommended for analysis [16,19].

- Ribs: We recorded whether there is a lack of ossification (by staining the sternebrae), and if numerary or not.

- Sternebrae: We record if there is a lack of ossification (according to the sternebrae staining), and whether it has merged or not
- Pasterns recorded whether there is a lack of ossification (according to the staining of the metacarpals), and the absence or not of metacarpals.
- Phalanges of forelimb: There was no lack of ossification if (according to the staining of the phalanges), and the lack or absence of phalanges.
- Hocks: We recorded whether there is a lack of ossification (as staining of metatarsals phalanges), and the absence or metatarsal.
- Phalanges of the hind: We recorded whether there is a lack of ossification (as phalanges staining), and the absence or phalanges.
- Sacral vertebrae: We recorded whether there is a lack of ossification (as staining of the phalanges), and the absence or not of these.

3. Results and discussion

3.1. Analysis in the rat reproductive harm parent

Table 2 presents the effect of treatment and the level of reproductive harm in female specimens of *R. norvegicus*. The total number of fetuses (134) and implants (89) concentration 0.5 mg/L is statistically significant, independent of treatment because not reflected an increase in the number of resorptions. Likewise, in Table 2 is shown the significant difference in total early resorptions the highest concentration

Table 2.

Effect of sodium arsenate (Na_2HAsO_4) and chromium trioxide (CrO_3) at reproductive harm in *R. norvegicus*

Variable	Control		Experimental treatments		
	Sucrose, %	CrO_3 , mg/L	Na_2HAsO_4 , mg/L		
	5	166	0.5	943.9	1887.9
N° Pregnant females	10	10	10	10	10
N° Total fetuses	88	111	134*	119	85
Rate fetuses/mother*	8.8 ± 2.4	11.1 ± 3.4	13.4 ± 2.7	11.9 ± 4.2	8.5 ± 3.2
N° Total implantations	89	113	136*	119	113
Relac. Implant./mother	8.0 ± 2.2	11.2 ± 3.3	13.6 ± 2.9	11.9 ± 4.2	11.3 ± 2.0
N° Total resorption tempreas	1	1	2	0	25**
Rate resorption tempreas /mother*	0.1 ± 1.3	0.1 ± 1.3	0.2 ± 0.4	0	2.5 ± 2.5
N° Total late resorptions	0	1	0	0	3
R ate late resorptions /mother*	0	0.1 ± 0.3	0	0	0.3 ± 0.9

Not:* = mean ± standard deviation; ** = Significant difference from control group, Dunnett's test , p<0.05 [23-24].

Source: Own elaboration

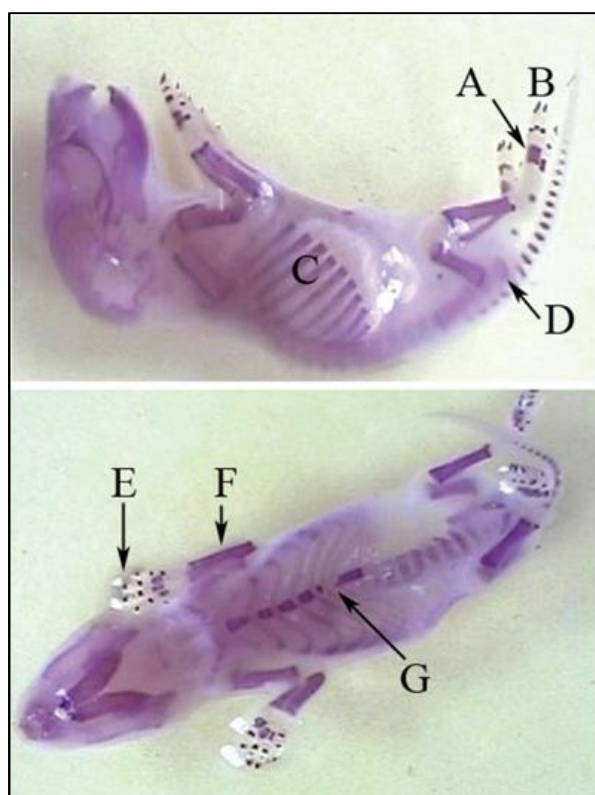


Figure 3. Major centers of ossification in rats. A. metatarsals; B. posterior phalanges, C. ribs, G. sternebrae, D. sacral vertebrae; E. previous phalanges; F. metacarpals.

Source: Own elaboration

(1887.9 mg/L), which indicates a toxic effect at the beginning of embryonic development, which is reflected in the total number of fetuses obtained in this concentration. It is particularly important to note that the reference teratogen CrO_3 , which has been reported in mice, shows no statistically significant harm pregnant females of *R. norvegicus* at the concentration tested.

3.2. Analyzing changes in the bone tissue in the fetus

Teratogenic effect in fetuses (Table 2), seven markers skeletal abnormalities with two variables each, statistically determined by Dunnett's test.

The lack of ossification in the relevant variable, being nonsignificant variables supernumerary ribs, fused sternebrae, metacarpals absence of previous phalanges, metatarsal, phalanges and sacral vertebrae later. To 166.00 mg/L of CrO_3 (Fig. 4B), 0.5 mg/L Na_2HAsO_4 (Fig. 4C) and 943.9 mg/L Na_2HAsO_4 (Fig. 4D) were statistically significant variables ossification faults in the ribs, sternebrae, metacarpals, phalanges above, metatarsals, phalanges and sacral posterior (Table 3).

For the highest concentration of Na_2HAsO_4 (1887.9 mg/L) (Fig. 4E), the variables that showed statistical significance were the lack of ossification in sternebrae, previous phalanges, phalanges later and sacral vertebrae (Table 3).

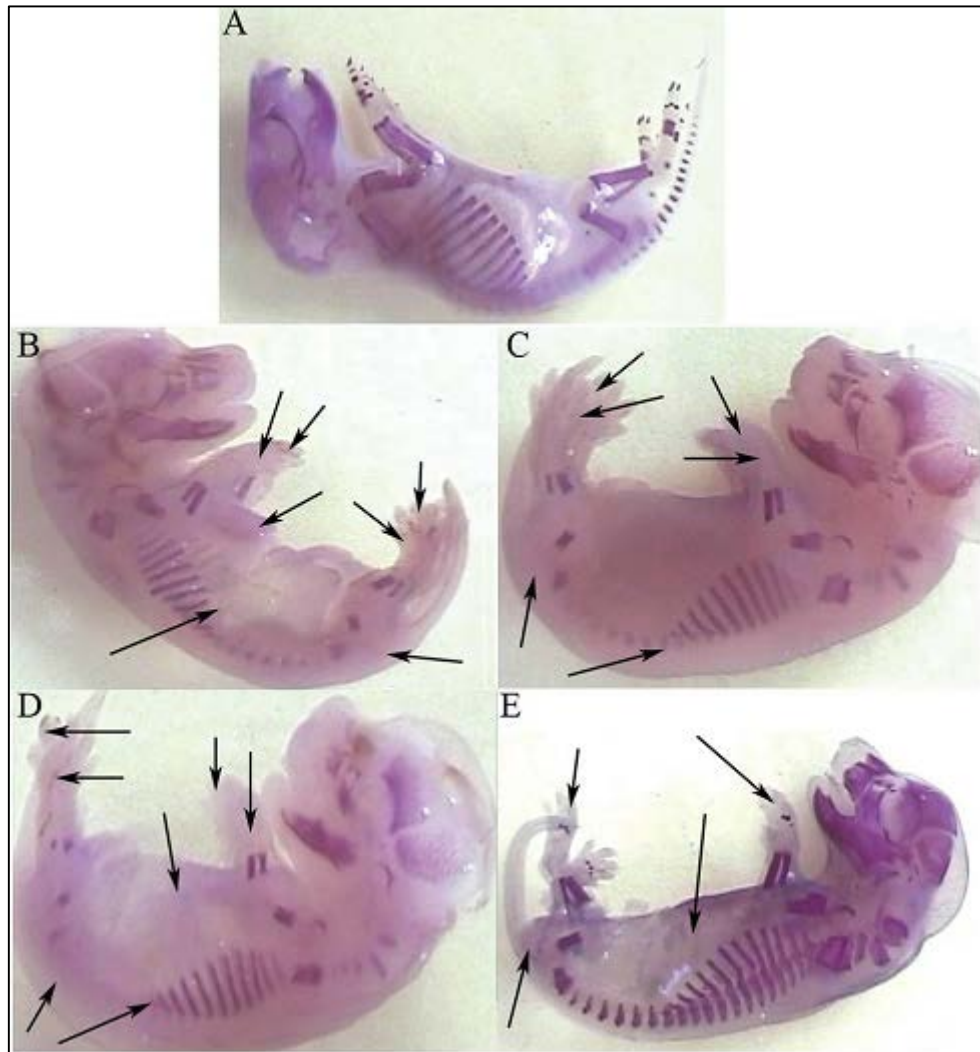


Figure 4. Effect of treatments on the process of ossification. A. Witness. B. Lack of ossification in ribs, sternebrae, metacarpals, phalanges above, metatarsals, phalanges sacral posterior and produced by administration of chromium trioxide (CrO_3). C. Lack of ossification in ribs, sternebrae, metacarpals, phalanges above, metatarsals, phalanges sacral posterior and produced by administration of 0.5 mg/L of sodium arsenate (Na_2HAsO_4). D. Lack of ossification in ribs, sternebrae, metacarpals, phalanges above, metatarsals, phalanges sacral posterior and produced by administration of 943.95 mg/L of sodium arsenate (Na_2HAsO_4). E. Lack of ossification in sternebrae, phalanges previous and subsequent phalanges sacral vertebrae, caused by administration of 1887.91 mg/L of sodium arsenate (Na_2HAsO_4). Note: Magnification is not the same in each photo; arrows indicate points without ossification. Source: Own elaboration

Based on the results, it was determined that Na_2HAsO_4 induces reproductive harm in female progenitors of *R. norvegicus* and teratogenic damage in fetuses, because it shows statistically significant toxicity in the rat mother to the highest concentration of 1887.9 mg/L, which was established based on the ratio of the number of implantations and the number of fetuses obtained.

Furthermore, it was determined that the toxic effect acting in the early stages of embryonic development, indicating that one of the possible causes of these Na_2HAsO_4 resorption is not a metabolite of it, and acting toxic level. Also, it demonstrates that there is a different sensitivity for each of the stages of embryonic development.

Teratogenic damage caused by Na_2HAsO_4 in fetuses is statistically significant in the process of ossification in the seven markers analyzed in two of the three concentrations tested (0.5 mg/L and 943.9 mg/L).

Being relatively important to mention that the lowest concentration is found in the water used for human consumption in Zimapan, Hidalgo. So the effect caused by the Na_2HAsO_4 can be associated with a social and health problem in this town.

This allows corroborating the mechanisms and factors described above that may affect the expression of a teratogenic effect and, therefore, the difficulty of its evaluation. The results can vary from one experimental organism to another, genetic susceptibility of each organism, by the influence of maternal genome because it may interact with the metabolism of other compounds that come from mother to fetus. And participate in the resistance to infection, biochemical and molecular processes that can affect the product. Another factor that can affect the susceptibility teratogenic response of organisms, which may vary between different stages of embryonic development at the time of

Table 3.

Effect of sodium arsenate (Na_2HAsO_4) and chromium trioxide (CrO_3) in the development of the fetal bone treated in the prenatal period

Skeletal abnormalities	Control		Experimental treatments		
	Sucrose, %	CrO_3 , mg/L	Na_2HAsO_4 , mg/L		
	5	166	0.5	943.9	1887.9
Total analyzed fetuses	85	85	85	85	85
*Ribs					
N° Supernumerical	0	1/1.17	0	2/2.35	2/2.35
*Ribs					
Lack ossification, N°/%	0	43/50.58*	21/24.70*	17/20.00*	5/5.88*
*Estenebras					
N° Merged	0	0	0	0	0
*Estenebras					
Lack ossification, N°/%	0	70/82.35*	49/57.64*	32/37.64*	50/8.82*
*Pastern					
N° Absence	0	0	0	0	0
*Pastern					
Lack ossification, N°/%	0	40/7.05*	30/35.29*	32/37.64*	8/9.41*
* Previous phalanges					
N° Absence	0	0	0	0	0
* Previous phalanges					
Lack ossification, N°/%	0	66/77.64*	56/65.88*	65/76.47%	48/56.47
*Hocks					
N° Absence	0	0	0	0	0
*Hocks					
Lack ossification, N°/%	0	40.47*	31/36.47	32/37.64	8/9.41
* Phalanges later					
N° Absence	0	0	0	0	0
* Phalanges later					
Lack ossification, N°/%	0	76/89.41*	75/88.23*	69/81.17*	66/77.64*
* Sacral vertebrae					
N° Absence	0	0	0	0	0
* Sacral vertebrae					
Lack ossification, N°/%	1/1.17	71/83.52*	76/89.49*	70/82.35*	69/81.17*

Note: * Significant difference from control group, Dunnett's test, $p < 0.05$; # number of exemplary [23-24].

Source: Own elaboration

exposure. In addition, we have to consider the different mechanisms of action that may arise in cells and tissues by different types of teratogens, hence the importance of selecting a good bioassay, concentration, route of administration and the time of exposure [20].

In terms of CrO_3 , its effect was tested on the positive control at the level of lack of ossification in the bone tissue at the concentration tested, which corroborates the sensitivity of the bioassay to a chemical agent. This emphasized the potential of CrO_3 to induce at least lack of ossification in the bone tissue and serves as an index for comparison with other chemical agents to determine effect [18].

Na_2HAsO_4 at the highest concentration used in this work, causes damage in rats progenitor player. Its effect on ossification was significant only in four of the seven markers (lack of ossification in sternebrae, previous phalanges, phalanges later and sacral vertebrae).

It is evident that these markers, called ossification centers are sensitive to Na_2HAsO_4 in the concentrations evaluated, while the other three markers (lack of ossification in the ribs, metacarpals and metatarsals) were not sensitive to this compound, that is, they show an apparent sensitivity different depending on the chemical and / or concentration evaluated [21-22]. There are reports of some heavy metals, although the fetal toxic effects expressed at high and low concentrations thereof, are capable of inducing alterations in several embryonic developments [23-24]. It is recommended to conduct more experiments at different concentrations and

with other compounds to assess the level of risk in human populations.

5. Conclusions

Teratogenic damage caused by Na_2HAsO_4 in fetuses is statistically significant in the process of ossification in the seven markers analyzed.

Based on these results, it is recommended further studies using Na_2HAsO_4 and try it in different kinds of organisms and trophic levels.

With this, it can be concluded that these markers, called ossification centers are sensitive to Na_2HAsO_4 in the concentrations analyzed. The other three markers (lack of ossification in ribs, metacarpals and metatarsals) are not sensitive to this compound

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