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Molecular Mechanism of Induction of Male Infertility in Lead-Exposed Rats

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ABSTRACT

Epidemiological studies of male workers with blood lead levels have shown that they increase the risk of infertility. Several studies on the reproductive system of male animals have also shown that lead is toxic to testicular function. However, the mode of action of lead on male reproductive functions remains poorly studied. The aim of this study was therefore to elucidate the mechanism by which lead induces male infertility. Ten male Wistar rats were divided into an experimental group (N=5) and a control group (N=5). The control group received sterile distilled water while the experimental group received a 0.3% lead acetate solution for 90 consecutive days. At the end of the experiment, the rats were euthanised. Blood was collected from the rats by cardiac puncture for the determination of malondialdehyde (MDA). The left testis was isolated for assessment of apoptosis using the Tunel method. Part of the right testis was used to assess the expression of apoptotic and oxidative genes by RT-PCR and the other part for the production of testicular homogenates. The results showed a highly significant increase in the concentration of MDA in the blood and testicular homogenates of rats from the experimental batch compared with rats from the control batch. The results also showed a highly significant increase in the number of apoptotic cells (p=0.0002) in rats from the experimental batch. This study highlights the mechanism by which lead induces male infertility. It shows that exposure to lead causes oxidative stress correlated with excessive cell proliferation due to under-expression of P53. This cell proliferation induces more abundant apoptosis of germ cells, which could explain the drop in male fertility.

Keywords

Gene expression, Lead, Male infertility, RT-PCR.

Introduction

In recent decades, the issue of declining sperm parameters has been the subject of increasing debate [1]. Decreased sperm counts have been reported, and were thought to be due to environmental and occupational exposure to various chemicals [2,3]. Since then, a great deal of work has been carried out in an attempt to establish a link between sperm function and environmental and occupational exposures.

Many authors have reported the harmful effects of environmental pollutants on sexual function, such as tobacco [4], pesticides [5] and heavy metals like lead [6], cadmium [7], and mercury [8]. Among these pollutants, lead appears to be one of the most abundant and widespread metals (water pipes, industrial pollution) in our environment. Its bioconcentration in seminal plasma has encouraged researchers to study its potential effects on reproductive function [5]. The risk is generally thought to be directly correlated with increasing concentrations and duration of exposure [9,10]. Epidemiological studies of male workers with blood lead levels have shown that they increase the risk of infertility [11,12].

In addition, several studies on the reproductive system of male animals have documented lead as a toxicant to testicular tissue and function such as significant reductions in epididymal sperm parameters in lead acetate administered in drinking water and

cessation of spermatogenesis in rats [13-15]. Macroscopic changes in accessory sex organs, such as decreased weight of testes, seminal vesicles, epididymis and ventral prostate, have been demonstrated in various studies using laboratory animals. However, the mode of action of this heavy metal on male reproductive functions remains poorly studied.

The aim of this study is therefore to elucidate the mechanism by which lead is thought to induce male infertility.

Materiel and Methods

Animal treatment

Ten (10) male rats with 12 weeks of the Wistar strain, were housed in a well-ventilated room with light and dark cycle of 12 h and under standard laboratory conditions. Animals had unrestricted access to rat chow and water. The animals were randomly distributed to experimental group (N=5) and control group (N=5). The control group received sterile distilled water while the experimental group received lead acetate solution with a concentration of 0.3 % (3 g/L) for 90 consecutive days. At the end of the experiment, rats were euthanized.

Animal ethics

The tests carried out on wistar rats in this study were conducted in accordance with the protocol in force at the Institut Pasteur de Côte d'Ivoire.

Experimental Design

Blood Collection and Tissue Preparation

Blood samples were taken from each animal by cardiac puncture immediately after death, and the plasma was separated and stored at -20ºC for malondialdehyde assays.

The testes were removed and stripped of their fatty tissue. The left testis was used for histological sections. 100 mg of the right testis from each rat was cut and used for the extraction of genetic material (RNA) and the other part was used to produce testicular homogenates.

Preparation of Testicular Samples

Testicular homogenates were prepared according to the method described by Kameni [16]. The right testis of each rat was removed from its albuginea and ground in a mortar on an ice tray. The crushed material was suspended in sodium phosphate buffer (0.1M; pH 6.8). After centrifugation at 10.000 rpm for 20 minutes at 4°C, the supernatant was collected and dispensed into Eppendorf-type conical tubes and stored at -20°C for the various tests.

Testicular and Blood Malondialdehyde Assay

Lipid peroxidation in plasma and testicular samples was assessed by malondialdehyde (MDA) assay. This assay was performed according to the method described by Hiroshi et al. [17]. Thus, 2 mL of 1.5% potassium chloride (KCl) was added to the samples made up of testicular and plasma mashes from animals. The resulting mixture was homogenized using a vortex. Then 1 mL of the homogenate was collected and 2.5 mL of 20% trichloroacetic acid was added. Subsequently, the mixture was centrifuged at 3500 rpm for 10 min at 4ºC. The resulting pellet was dissolved in hemolysis tubes containing 2 mL of 0.05 M concentration sulphuric acid (H_2SO_4) and 3 mL of 2 M molarity thiobarbituric acid. The tubes were incubated in a hot water bath (60ºC) for 30 minutes. The contents of the tubes were cooled to room temperature and the colour highlighted by adding 4 mL of n-butanol. Finally, the intensity of the coloration was determined by spectrophotometry (*Spectro Analyti Kjena Specord 210 plus*). The quantities of MDA formed are obtained using Beer-Lamber's law.

TUNEL Analysis

After histological sections had been prepared using the technique described by Tarabishy et al. [18] testicular, tissues two micrometres thick were deparaffinised, rehydrated and soaked in proteinase K (20 mg/ml) at 37°C for 180 minutes in order to permeabilise the sections. Apoptotic DNA fragmentation was assessed using the TUNEL method on these deparaffinised rat testis sections. The TUNEL Chromogenic Apoptosis Detection kit was used according to the manufacturer's instructions (**Genecopoeia, USA**). Samples were observed under phase contrast (**Olimpus, CK41SF, Philippine**) and micrographs were taken. Germ cells were quantified in each tissue section by counting the number of TUNEL-positive cells in random areas. Cells shown in black are TUNEL-positive.

Total RNA Isolation and Quantification

Total RNA was extracted from rat testes using the phenol-chloroform method described by Lee et al. [19]. DNAse I was used to avoid contamination of the extracted RNA by DNA. RNA quality was tested by agarose gel electrophoresis and RNA concentration was determined using the Quibit (**3.0 Fluorometer, Malaysia**).

Quantitative reverse transcription PCR (qRT-PCR)

RNA extracted from testicular tissue was transcribed into cDNA using the reverse transcription system (**TAKARA, Japan**) following the manufacturer's instructions. Sequence amplification for gene expression evaluation required the use of primers (**Sigma, Chemical Company, USA**).

Statistical Analysis

Statistical analysis was carried out by the use of GraphPad Prism 5.0. Data are expressed as means SD and $p < 0.05$ was considered statistically significant.

Results

MDA dosage

MDA was assessed in the blood and testicular homogenates of rats from both batches (control and experimental). The results showed that the blood of rats from the experimental batch contained 13.7 ± 0.75 µg/L compared with 11.1 ± 0.50 µg/L in rats from the control batch. The results obtained indicate that lead induced a significant increase ($p = 0.0193$) in the concentration of MDA in the blood of rats from the experimental batch compared with rats from the control batch (Figure 1).

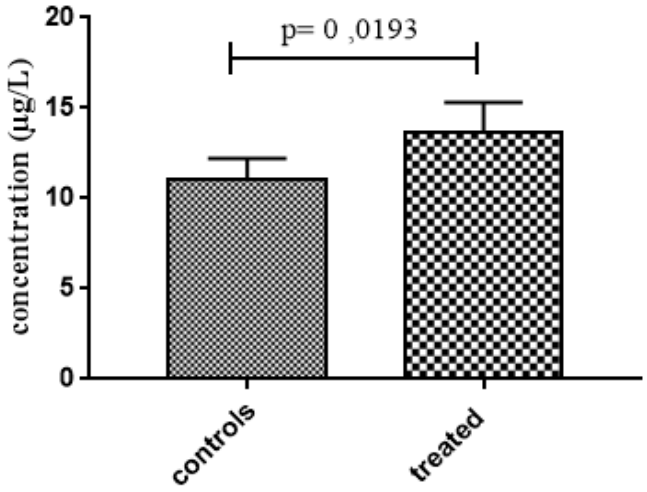

Figure 1: Blood MDA level.

In addition, the results obtained show that lead induced a highly significant increase (p = 0.0014) in the concentration of MDA in the testicular homogenates of rats from the experimental batch compared with rats from the control batch (Figure 2). MDA was measured at 33 ± 2.17 µg/L in the testicular homogenates of rats from the control group compared with 59 ± 5.03 µg/L in rats from the experimental group.

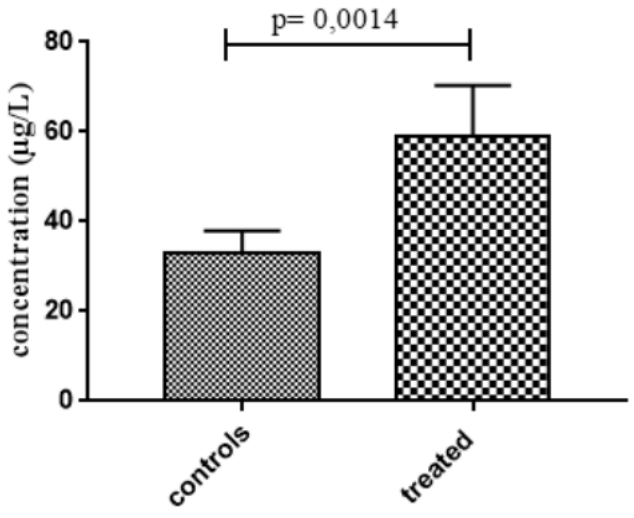

Figure 2: Testicular MDA level.

Testicular Apoptosis

Germ cell apoptosis was assessed by histo-immunochemistry. Figure 3 shows the apoptotic cells of rats from the control batch (Figure 3A) and the experimental batch (Figure 3B). Malassez cell count revealed 10.4% ± 1.04 apoptotic cells in the seminiferous tubules of rats from the control group compared with 23.80% ± 1.74 in the seminiferous tubules of rats from the experimental group (Figure 3C).

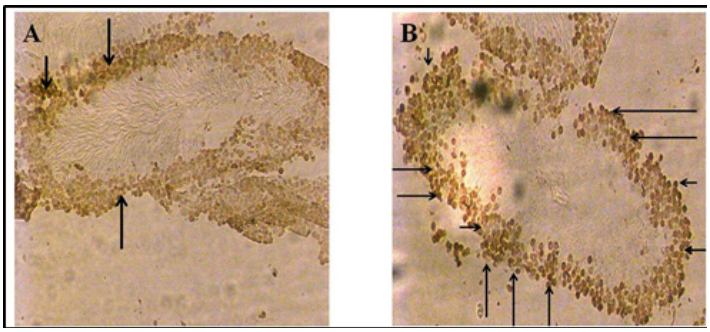

Figure 3: A: Micrograph showing apoptotic cells in control rats. B: Micrograph showing apoptotic cells in experimental rats, Arrows indicate apoptotic cells.

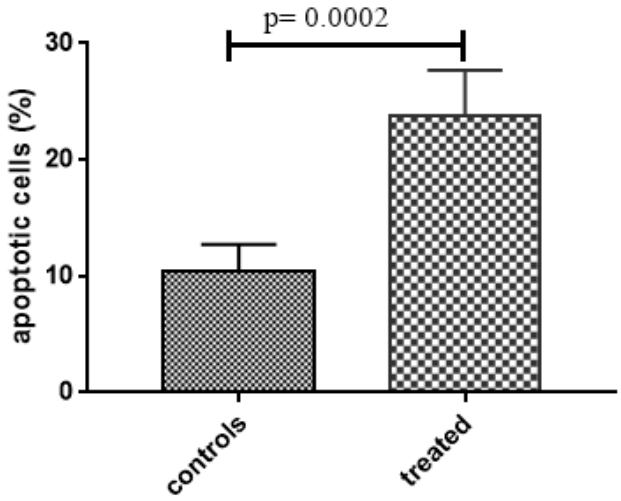

Figure 3C: Testicular germ cell apoptotic count.

Lead induced a highly significant increase (p= 0.0002) in the number of apoptotic cells in rats from the experimental batch compared with rats from the control batch.

Testicular Apoptosis and DNA Damage

Evaluation of the expression of genes involved in the regulation of apoptosis revealed a decrease in the expression of the P53, Bcl-2 and caspase-3 genes in the testicular homogenates of rats from the experimental batch compared with the control batch. On the other hand, a very significant increase in the testicular expression of TNFα was observed in the testicular homogenates of the experimental batch compared with the control batch (Figure 4).

Testicular Oxydative Stress

Evaluation of the expression of antioxidant enzymes in the testes of rats from the two batches in this study revealed a significant increase in the expression of superoxide dismutase (SOD) and a significant reduction in glutathione peroxidase (GPx). At the same time, it was observed that there was no variation in the expression of catalase (Figure 5).

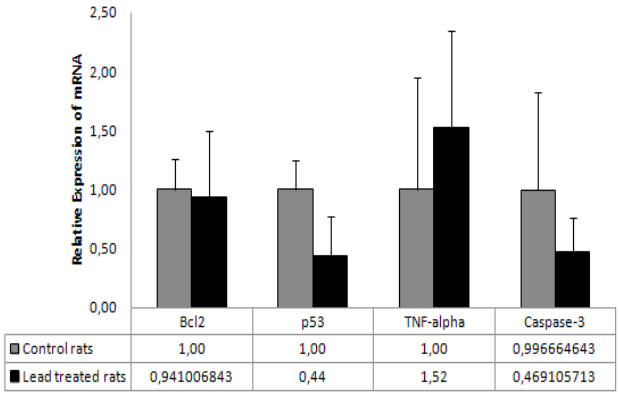

Figure 4: Apoptotic gene expression.

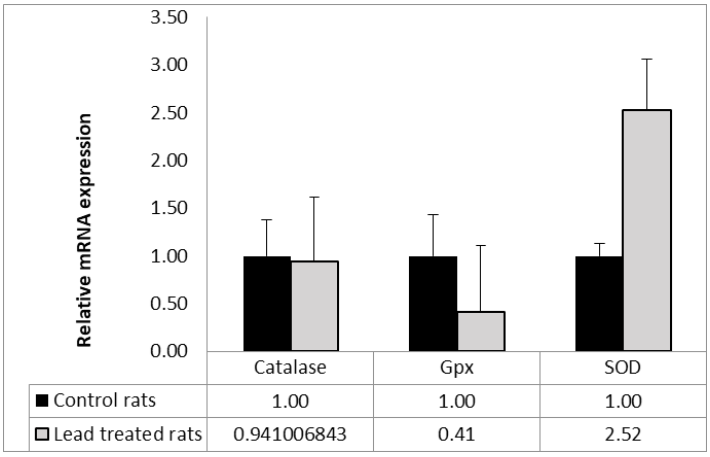

Figure 5: Antioxidant enzyme expression.

Table 1: Sequences of pro- and anti-apoptotic primers used.

Targets	Séquences	Tº d'hybridation
Bcl_2 : forward	**5′-CTGGTGGACAACATCGCTCTG**	68,1 ºC
Bcl_2 : reverse	**5′-GGTCTGCTGACCTCACTTGTG**	65 ºC
P53 : forward	**5′-CCAAGAAGGGCCAGTCTACGT**	66,4 ºC
P53 : reverse	**5′-GCCCCACTTTCTTGATCATTG**	65,5 ºC
TNFα : forward	**5′-GCATGATCCGAGATGTGGAA**	66,1 ºC
TNFα : reverse	**5′-GGCTGACTTTCTCCTGGTATG**	62,6 ºC
Caspase-3 : forward	**5′-TACCCTGAAATGGGCTTGTGT**	65,4 ºC
Caspase-3 reverse	**5′GTTAACACGAGTGAGGATGTG**	59,7 ºC

Table 2: Sequences of antioxidant primers used.

Targets	Sequences	Hybridization Tº
SOD : forward	**5′-GGTCCACCTCGAACTACTTTATG**	62,5ºC
SOD : reverse	**5′-GGTGATCAGGAACATGGAATCT**	64ºC
CAT : forward	**5′-CTCAGGTGCGGACATTCTATAC**	62,4ºC
CAT : reverse	**5′-GACTCCATCCAGCGATGATTAC**	64,6ºC
GPx : forward	**5′-CAGTTCGGACATCAGGAGAATG**	65,8ºC
GPx : reverse	**5′-AGCCATCACCAAGCCAATAC**	63,8ºC

SOD **:** Superoxide dismutase; CAT : Catalase ; GPx : Gluthation peroxidase Tº : Temperature

Discussion

In this study, in order to understand the mechanism by which lead reduced male fertility, the expression of enzymatic markers of oxidative stress (SOD, Catalase and GPx), as well as that of apoptosis-regulating proteins, in this case P53, Caspase-3, Bcl-2 and TNFα, was assessed by RT-PCR.

Molecular analysis of the proteins involved in regulating apoptosis revealed that the P53 protein was underexpressed in the testes of lead-exposed rats. P53, the guardian of the cell cycle [20], is a tumour suppressor protein. Its loss therefore predisposes to various types of tumour [21,22]. However, its role in spermatogenesis and fertility is less well known [23]. In fact, studies by Beaumer et al. [24] revealed that mutated P53 transgenic mice are fertile and show no major abnormalities in spermatogenesis apart from an increase in the number of spermatogonia. However, under-expression or loss of P53 leads to abnormal cell proliferation. The work of Jacks et al. [25] reported an increase in the number of spermatogonia at the spermatocyte I stage, suggesting excessive division at this stage of the spermatogenic cycle. This excessive proliferation of cells induces activation of a cell regulation system, in particular apoptosis. Apoptosis is defined as a phenomenon of programmed cell death [26]. It therefore occurs normally in all cells, either to eliminate defective cells or to maintain cellular homeostasis [27].

Consequently, the under-expression of P53 observed in this study would lead to an increase in spermatogonia and could explain the significantly high number of apoptotic cells observed in the seminiferous tubules of rats in the experimental batch. Although apoptosis occurs normally in all cells, certain stimuli or conditions can trigger more abundant apoptosis, as Jacobson et al. [28] agree.

The results of the present study are consistent with this hypothesis. The TNFα is over-expressed in the testes of rats exposed to lead. According to Pei-Li [29], the TNFα protein is a cell death receptor. It is therefore a key marker for the molecular assessment of apoptosis in mammalian cells [30]. TNFα also regulates numerous biological processes, including cell growth, differentiation, activation and apoptosis [31].

In addition, the results of this work show underexpression of the Bcl-2 protein. According to Czabotar et al. [32] cell death factors are regulated by Bcl-2 family proteins. This underexpression of the Bcl-2 protein would explain the overexpression of TNFα, the pro-apoptotic protein evaluated in this study. Several authors have pointed out the anti-apoptotic role of Bcl-2 [33,34].

According to Raûl and Ricardo [35] Bcl-2 interacts with pro-apoptotic proteins to repress the phenomenon of apoptosis. Several caspases exist but the key caspase involved in apoptosis is caspase-3 [27]. Caspase-3 is a protease that regulates the process of apoptosis. Under-expression of this gene indicates a failure in the regulatory process and suggests an increase in programmed cell death. The results of this study, which showed that caspase-3 was underexpressed in the experimental batch of rats, are consistent with this hypothesis. These results of molecular analysis were confirmed by those of the histo-immunochemical approach. The results show several apoptotic germ cells at the periphery of the seminiferous tubules of rats given drinking water containing lead acetate. These germ cell deaths by apoptosis in the early stages of

spermatogenesis could explain the reductions in sperm parameters and spermatogenic disturbances observed by several research teams that have exposed rodents to lead [6,13]. It has been shown that germ cell apoptosis plays an important role in controlling sperm production in many species and is linked to male infertility [36].

These changes in the expression of apoptosis-regulating proteins could be due to oxidative stress. According to Lysiak et al. [37] oxidative stress is associated with germ cell apoptosis in rodent testes. The results of this study suggest a higher level of oxidative stress in the rats of the experimental batch. Malondialdehyde (MDA), the main product of oxidative stress is higher in the testes of rats exposed to lead. This observation was confirmed by analysis of the expression of enzymatic markers. In the course of this work, SOD, the main enzyme involved in combating free radicals in spermatozoa [38], was over-expressed. Finally, the results of this study showed that the expression of catalase in rats from the experimental batch appeared to be identical to that in rats from the control batch. According to Griveau and Lannou [38], catalase has low activity. This observation was confirmed in the present study.

Conclusion

This study highlights the mechanism by which lead induces male infertility. It shows that exposure to this heavy metal causes oxidative stress. This oxidative stress is correlated with excessive proliferation of male germ cells due to under-expression of P53. This cell proliferation leads to more abundant apoptosis, as evidenced by overexpression of TNFα and underexpression of Bcl-2. This excessive apoptosis of cells in the early stages of spermatogenesis could explain the reduced number of spermatozoa reaching maturity.

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