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Protective Role of Withaferin A on Lead Acetate Induced Testicular Toxicity - Histopathological and Immunohistochemical Analysis in Wistar Rats

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Abstract

Infertility is increasing and becoming a major concern, particularly in a nation like India where there are large populations on the one hand and childless couples on the other. Male infertility affects that half of the population. A number of factors contribute to infertility, some of which are environmental in nature. One of such notable factor of environmental pollutant is the lead. Lead is almost present in all of your surroundings in different ways, especially usage of lead in the paint industries, lead pipes and in toys. Its effect is detrimental to reproductive organs especially to testis. To treat this issue withaferin A is used to scientifically validate its fertility enhancing property. A total of five sets of Wistar rats were obtained. Control, lead acetate (Pb), lead acetate + withaferin A, lead acetate + vitamin A & selenium and withaferin A alone, comprise Group I, II, III, IV and V respectively. Following the experiment, rats were euthanized and samples were collected for histopathological examination. Group I and Group V animals in the control and drug control group exhibit no alterations. After being exposed to lead acetate solution and inflicting several reproductive cell damages, Group II undergoes a substantial pathological alteration. Withaferin A considerably improves the lead induced toxic condition of testis than vitamin A and selenium treated group. Alternate approach makes substantial use of the withaferin A in animal models of reproductive cell damage caused by oxidative stress upon lead toxicity. Thus, withaferin A have encouraging outcomes on male infertility.

Keywords: *Antioxidants, Histopathology, Lead Toxicity, Male Infertility, Testicular Damage, Withaferin A.*

Introduction

Infertility affects 186 million people and 48 million couples, according to data from the World Health Organization (WHO). According to WHO (2018), infertility is defined as a condition of the male or female reproductive system that results in the inability to achieve pregnancy after one year or more of sexual activity [1]. There is lead everywhere, and people use lead-containing products on a daily basis. Paints, cosmetics, plumbing supplies, and leaded petroleum products all contain lead. The need for lead mining, smelting, and recycling is increased by its use in batteries [2]. Lead is the primary material used to make batteries, and its widespread use is a result of its affordable and effective electrical surges. Water composite of lead, cosmetics, and herbal medication consumed by the general people. It was discovered that

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heavy metals and industrial pollutants provide a health risk to the reproductive system [3].

Lead is a significant heavy metal found naturally in the earth [4]. Lead levels in the blood rose almost one-fold higher in the comparative study between fertile and infertile men [5]. Male workers' blood lead levels range from 10 to 40 μg/dl, according to an epidemiological studies. The study highlight the higher risk of infertility brought on by lead exposure. Another investigation on male workers reported higher blood levels above 25 μg/dl of lead revealed fewer offspring than control participants [6]. Lead was shown to be an active factor in male reproductive abnormalities in rats in animal experiments [7]. According to the investigations, the sperm count and concentration of exposed individuals are significantly lower [8]. It was discovered that the amount of ejaculation had decreased [9]. Testicular tissue and function abnormalities are evident in animal investigations [10]. Mice given lead acetate had fewer spermatozoa and rats' spermatogenesis was completely halted [11]. Rats subjected to lab experiments displayed notable organ changes, such as reduced weight in the testes, prostate, seminal vesicles, and epididymis. The animals' reproductive systems also showed histological pathological alterations. At ≤10 μg/dl of lead in blood, lead is negatively affecting motility and viability [12].

A well-known heavy metal with hazardous qualities, lead (Pb) is utilised in many different ways all throughout the world, particularly in developing nations. It is widely utilised in a wide range of items in India, including batteries, leaded paints, leaded gasoline, and the list goes on. A particularly sensitive group is youngsters because of the use of paints. Lead poisoning is caused by the childhood habit of licking doors and ingesting lead particles. In 1920, lead was introduced into petrol to lessen engine knocking and wear and tear. Workers in the sector become ill after being exposed to lead. This opened people's eyes to the need to reduce lead exposure, but because of its ductility and anticorrosive qualities, lead was still used in industries. Its continued use is a result of all these benefits over lead. Leaded petrol was removed from the market after 1970 and was outlawed in 1996. Within the respirable range, airborne particles can readily reach the metabolism and have detrimental effects. Lead and cadmium, the two most prevalent airborne components, are significant sources of airborne heavy metals in industrialized areas [13].

The male reproductive system is where lead and cadmium build up [14]. Men exposed to industrial aerosols of lead had lower sperm counts, delayed conception, and smaller families, according to research published earlier. In this study, lead levels were greater in the seminal plasma and blood plasma of workers who were exposed to lead. The concentration of lead in seminal plasma and blood plasma is positively correlated [15]. Due to the longer period of lead exposure at work, factory workers have low levels of infertility [9].

Together, the terms "withan" from the genus Withania and "olide," which is the scientific name for the lactone molecule, create the term "withanolides." In 1965, Lavie and their team isolated the first withanolide from *W. somnifera* was withaferin A [16]. Since then, 58 solanaceous plants from 22 genera have yielded more than 400 withanolides [17]. The presence of secondary metabolites, known as withanolides, which are formed via mevalonic and non-mevalonate pathways with a 3:1 proportion, is what gives *Withania somnifera* its biological characteristics [18]. Withaferin A, withasomniferin A, 5-dihydroxy withanolide, withanolides I–VII, withanone, withanolide A, withanolide D, oxygenated and sulfated withanolide, somniferin, somniferinin, tropine,

and pseudotropine are the primary chemical components of *Withania somnifera* [19].

Vitamin E was first identified by Evans and Bishop in 1922 as an antisterility factor known as X [20]. A potent supplement to boost the rate of reproduction is vitamin E. Its capacity to scavenge reactive oxygen species (ROS) was discovered through additional research [21].

Selenium is an effective antioxidant that can balance the system's homeostasis and lessen the effects of other antioxidants. Since its discovery by Swedish chemist Jacob Berzelius in 1817, selenium has been regarded as a necessary trace element for living things [22]. A crucial element in proteins that are encoded by the TGA codon is selenocysteine. According to Soudani et al. (2011), selenium is one of the essential micronutrients for humans. It contributes significantly to the antioxidant defence system [23]. According to earlier research, a lack of selenium can cause a number of illnesses. Since it shields cells from the damaging effects of free radicals, it is often regarded as being extremely vital for human health. Free radicals are changed into stable molecules by selenium compounds [24].

Materials and Methods

Study Approval

Saveetha Institute of Medical and Technical Sciences' Institutional Animal Ethics Committee gave its approval to the experiment (Approval No: BRULAC/SDCH/SIMATS/IAEC/12-2019/044). In order to guarantee the humane care and wellbeing of research animals, the study complied with the national rules set forth by the Committee for the Control and Supervision of Experiments on Animals (CCSEA).

Chemicals Used

Sigma Chemical Company, based in St. Louis, Missouri, USA, supplied the analytical-grade lead acetate, selenium, and vitamin E. Cayman Chemicals in the United States provided the active component, withaferin A. Sisco Research Laboratory (SRL) Private Limited in India was the supplier of additional lab chemicals and supplies.

Animal Maintenance

Wistar albino males weighing between 180 and 200 grams were kept in laboratory settings with controlled lighting and temperature (22–25° Celsius). The animals were kept in a light-dark cycle of 12:12. The rats were given a regular pellet diet and unlimited access to tap water.

Animal Grouping and Dosage

Animals were randomly segregated into 5 groups. Each group consists of 6 animals. Group – I: Control, Group – II: Lead acetate, Group – III: Lead acetate + Withaferin A, Group – IV: Lead Acetate + Vitamin E & Selenium, Group – V: Withaferin A.

Lead toxicity was induced to healthy adult rats that received lead acetate for 70 days of drinking water with 819 mg/L of lead (0.15% lead acetate) by oral route daily [25]. For the three animal groups, water containing lead acetate is maintained in place of the usual water diet (Groups II, III & IV). Water feeding with lead was continued regularly till 70 days are completed.

Vitamin E was given at a dose of 80 mg/kg body weight by oral route and selenium was supplemented at a dose of 1.6 mg/kg body weight by oral route daily for 28 days [26].

Histopathological Processing

Haematoxylin & Eosin

After 60 days of the total experimental period, animals were sacrificed by CO_2 in a closed chamber. Testicular tissues were dissected out and fixed in 4% paraformaldehyde and processed for histopathological analysis. The fixed testis

were hydrated sufficiently, dehydrated in alcohol, embedded with paraffin and sectioned in a Rotary microtome following standard histological techniques. Using standard Haematoxylin & Eosin staining, paraffin slices of 5 µm in thickness were examined under a light microscope.

Immunohistochemical Processing

For Immunohistochemistry, 4% paraformaldehyde fixed paraffin embedded brain tissue sections of 5 µm, mounted on a glass slide underwent heat-induced epitope retrieval in citrate buffer (pH 6.0) at 95°C for 20 minutes to unmask Caspase-3 antigens, enhancing antibody binding. After cooling, sections are incubated in 5% normal bovine serum albumin for 1 hour to block non-specific binding sites. Tissue sections are incubated overnight at 4°C with anti-caspase-3 primary antibody (dilution 1:200) for specific binding. Sections are treated with a biotinylated secondary antibody conjugated with HRP (Horse Radish Per-oxidase) to 30 minutes for signal amplification. DAB is applied as the chromogen, producing a brown precipitate on caspase-3 positive cells, followed by haematoxylin nuclear counterstaining for contrast. Slides are dehydrated, cleared and cover-slipped with a permanent mountant. Slides are analysed using bright-field microscopy and photographed.

Statistical Analysis

The findings were presented in the form of mean ± SD. In GraphPad Prism software version 7, a one-way analysis of variance (ANOVA) and Tukey's test were used for statistical analysis. Significant values were defined as $p < 0.05$.

Results

Histopathology of the Testis

Normal histoarchitecture of the testis with proper seminiferous tubules containing intact seminiferous epithelium was seen in Group-I Control rats. Also the seminiferous epithelium showed regular spermatogenesis. All spermatogenic cells were clearly suspended by the nourishing sertoli cells. No signs of cellular damage were seen. Whereas, the animals induced with lead acetate in Group-II, showed damaged seminiferous epithelium, impaired spermatagonial cells, vacuolization in sertoli cells and widened interstitial space with fibrosis. Also Group-II, showed the detrimental effects of lead acetate on the testis. In the lead acetate treated group, prominent damage to Leydig cells was found. Reduction of spermatogonia, spermatocytes, and spermatids in the seminiferous tubules of the testis was also noted. The rats induced with lead acetate and treated with Withaferin A, revealed remarkable recovery from all cellular damages and showed accelerated healing from lead induced toxic effects. There was comparatively less damage in the Leydig cells and showed promising results of improvement upon treatment with Withaferin A. The standard positive control rats of Group-IV, Lead acetate induced Vitamin E and Selenium treated animals depicted reduced cellular damages compared to Group-II. The drug Control rats of Group-V which was administered only with Withaferin A portrayed good cellular architecture with no signs of histopathology. Figure 1 shows the histopathology of testis stained with H&E.

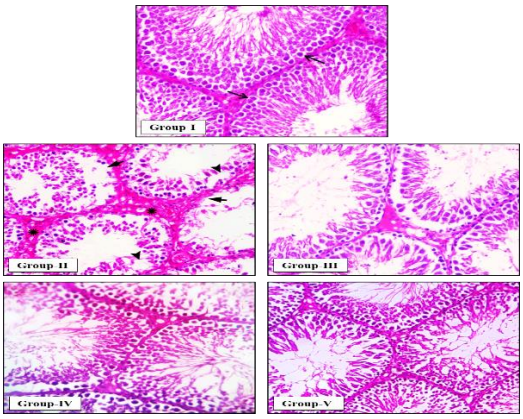

Figure 1. Photomicrograph Showing Histopathology of Testis Stained with H&E of Group-I: Control (Normal Saline); Group-II: Lead Acetate (Pb); Group-III: Lead Acetate + Withaferin A; Group-IV: Lead Acetate + Vitamin A & Selenium; Group-V: Withaferin A alone (Drug Control) at 20X Magnification. Thin Arrows – Normal Seminiferous Epithelium; Arrows – Damaged Seminiferous Epithelium without Spermatagonial Cells; Arrow-Heads – Vacuolization in Sertoli Cells; Asterisks – Widened Interstitial Space with Fibrosis.

Immunohistochemistry of the Testis

Immunohistochemistry analysis revealed strong and widespread expression of caspase-9 in the lead acetate induced rats (Group-II) when compared to control rats (Group-I). Intense immune-reactivity with clearly defined positive brown staining indicated oxidative imbalance mediated apoptosis with over expression of caspases-9. There were few spermatogonial cells with week expression of caspase-9 protein and only few cells with diminished staining intensity in the testis of lead acetate induced rat treated with Withaferin A (Group-III) and rats treated with vitamin E and Selenium (Group-IV), when compared to lead acetate induced rats (Group-II). Among these 2 groups Group-III showed less immune-reactive positive brown colored cells than Group-IV. The drug control group (Group-V) has meager or nil caspase-9 expression like control rats (Group-I). Figure 2 shows the immunohistochemistry localization of Caspase-3 in testis.

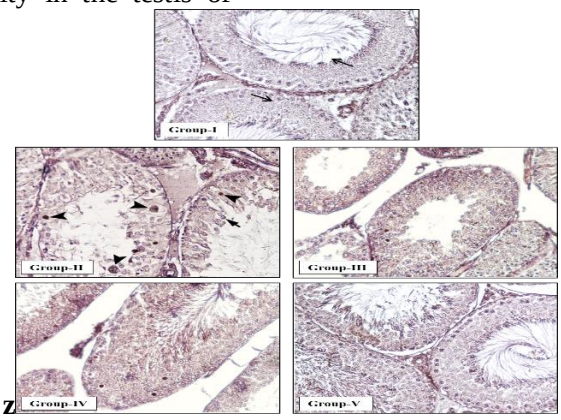

Figure 2. Photomicrograph showing Immunohistochemistry Localization of Caspase-3 in Testis Stained by DAB method in control and experimental groups. Group-I: Control (Normal saline); Group-II: Lead acetate (Pb); Group-III: Lead acetate + Withaferin A; Group-IV: Lead acetate + Vitamin A & Selenium; Group-V: Withaferin A alone (Drug Control) at 20X Magnification. Arrow-heads indicating Caspase-3 positive cells with dark brown coloration. Thin arrows indicating Caspase-3 negative cells with purple coloration (Normal cells). Arrow indicating apoptotic pyknotic cells. Caspase-3 positive neuronal expression is up-regulated in Group-II.

Histopathology of the Liver

Normal histoarchitecture of the liver was observed in the control (Group-I) and drug control groups (Group-V). Group II, showed the damaging effects of lead acetate on liver like prominent destruction of hepatocytes and hepatic steatosis. Due to the cytotoxic hepatocellular injury, zone based hepatic degeneration, focal and non-zonal apoptosis was also noticed. The histomorphology of the liver of the lead acetate intoxicated group treated with Withaferin A (Group-III) depicted comparatively less damage in the hepatocytes and showed promising results of improvement upon treatment with Withaferin A. Group-IV, showed the histopathology of the treatment group with Vitamin E and Selenium, where few damaged hepatic cells with scattered steatosis was seen. In our study, findings concluded that the liver cells were able to regenerate and prevent further tissue loss after being treated with Withaferin A. Figure 3 shows the histopathology of liver stained with H&E.

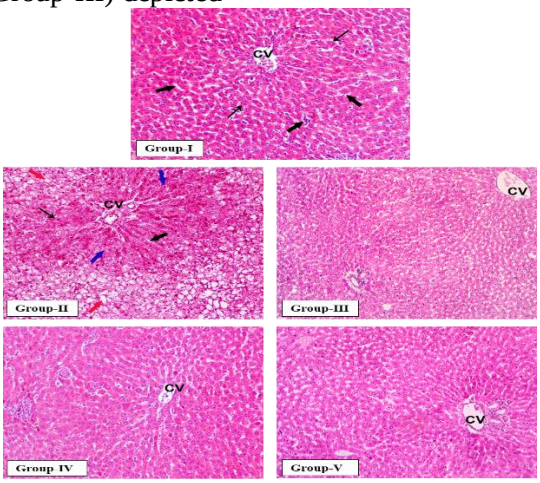

Figure 3. Photomicrograph Showing Histopathology of Liver Stained with H&E of Group-I: Control (Normal saline); Group-II: Lead acetate (Pb); Group-III: Lead acetate + Withaferin A; Group-IV: Lead acetate + Vitamin A & Selenium; Group-V: Withaferin A alone (Drug Control) at 10X Magnification. Thin arrows – Hepatocytes; Black thick arrows – Sinusoids; Blue thick arrows – Damaged hepatocytes; Red thick arrows – Microvesicular steatosis; CV – Central vein.

Histopathology of the Kidney

Normal histoarchitecture of the kidney was observed in the control (Group-I) and drug control groups (Group-V). Group II, showed the damaging effects of lead acetate on kidney like noticeable demolition of glomerulus, proximal convoluted tubules, distal convoluted tubules and with increased renal steatosis. Due to the cytotoxic effect of lead on renal tissue, cellular degeneration, zonal and focal apoptosis was also noticed. The histomorphology of kidney of the lead acetate induced withaferin A treated group (Group-III) represented comparatively less damage in the renal cortex and showed promising results of improvement upon treatment with Withaferin A preserving cellular function. Group-IV, showed the histopathology of the treatment group with Vitamin E and Selenium, where few damaged hepatic cells with scattered steatosis was seen. In our study findings conclude the nephrons, and tubules were regenerating after being treated with Withaferin A. Figure 4 shows the histopathology of kidney stained with H&E.

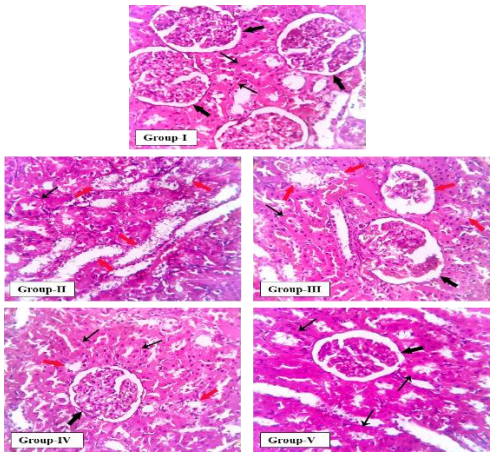

Figure 4. Photomicrograph Showing Histopathology of Kidney Stained with H&E of Group-I: Control (Normal saline); Group-II: Lead acetate (Pb); Group-III: Lead acetate + Withaferin A; Group-IV: Lead acetate + Vitamin A & Selenium; Group-V: Withaferin A alone (Drug Control) at 20X Magnification. Thin arrows – Proximal convoluted tubules; Black thick arrows – Glomerulus; Red thick arrows – Damaged tubules showing renal micro vesicular steatosis.

Discussion

The present study evaluated the deleterious effects of lead acetate primary male reproductive organ, the testis and subsequently treated with Withaferin A, showed remarkable improvement in testicular pathology regaining normal testicular architecture with enhanced spermatogenesis. Histological and immunohistochemical analysis revealed inflammation, altered spermatogenesis, apoptotic pathology with increased caspases-9 expression in testis of lead induced rats of Group-II. Whereas the rats showed decreased pathological features upon Withaferin A treatment of Group-III than positive control rats (Group-IV) treated with Selenium and Vitamin-E [26, 27]. Moreover Withaferin A treatment does not cause any hepato-renal pathology in liver and testis. The lead induced hepatotoxicity and nephrotoxicity is visible in the histopathology of liver and kidney. These cellular alterations was reduced in Withaferin A treated rats, the Group-III [28-30].

According to Neto et al. (2016), oxidative stress is a key tool in all diseases and is a defining hallmark of male infertility [44]. By upsetting the balance between antioxidants and reactive oxygen species (ROS), lead toxicant-induced oxidative stress seriously damages sperm quality, leading to abnormalities in spermatogenesis and male infertility [31]. Like the majority of other divalent metals, lead is typically bound to albumin, enzymes, short peptides, cysteine, methionine, and selenomethionine in tissues through ionic (in skeletal minerals) or coordination connections [32-34].

One important antioxidant that can aid in the elimination of reactive oxygen species is glutathione and it is one of the most extensively explored cell antioxidants at the moment which is a common thiol-containing tripeptide made up of glycine, L-cysteine, and L-glutamic acid [35, 36]. Like other divalent metals, lead can exit cells and circulate in serum or lymph after binding to glutathione (GSH). Damage to tissues result from the additional deposition of lead in due course of time [37]. ROS are thought to negatively impact key steps in the steroidogenic pathway, which converts cholesterol into steroid hormones [38]. Elevated reactive oxygen species levels cause damage to the acrosomal membranes, inactivation of glycolytic enzymes, and decreased sperm motility. Thus,

these factors render the sperm cell inoperable [39].

According to recent data, oxidative stress may play a role in acute liver damage, and certain antioxidants may help to lessen liver damage [40]. All of them contribute significantly to the development of both acute and long-term oxidative stress-induced liver damage. The focus of research is on inexpensive, easily accessible natural medicine made from plant-based chemicals that have antioxidant qualities. hence reducing the harmful effects that toxins have on people. *Withania somnifera*, sesame oil, *Moringa oleifera*, grape seed extract, *Vitis vinifera*, aqueous garlic extract, *Ficus carica*, *Pongamia pinnata* flowers, and many more plant extracts may provide protection against toxicity. To lessen the harmful effects of toxicants on both humans and animals, there has been a constant search for easily accessible, affordable phytochelators or natural antidotes with antioxidant qualities. Thus, it has been well validated that plant extracts shield experimental animals from the toxicity caused by lead.

Our research demonstrated that in response to lead acetate-induced testicular toxicity, the antioxidant activity, anti-inflammatory characteristic, and cell-nourishing capacity of Withaferin A restored and preserved the testicular oxidative balancing mechanism by increasing cell survival and further resupplying the hypothalamo-hypophyseal-testicular axis for controlling testicular function. A thorough understanding of the molecular regulation of the active compound on lead based testicular toxicity can be carried out in future studies which might have a clear research scope on its pathophysiology.

Conclusion

From the examined histopathological characteristics that show positive results in our current study, we may conclude that our findings provide a favourable outcome in reverting damage on the testicular organ. Therefore, it is possible to hypothesise that Withaferin A's protective action against lead acetate-induced testicular toxicity is due to its antioxidant qualities and capacity to scavenge reactive oxygen species.

Author Contributions

Author 1: Rajkumar. D, carried out the study by collecting data and drafted the manuscript after performing the necessary statistical analysis and in the preparation of the manuscript.

Author 2: Karthik Ganesh Mohanraj, aided in conception of the topic, designing the study and supervision of the study, statistical analysis, correction and final approval of the manuscript.

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Source of Funding

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Conflicts of Interest

There is no conflict of interest to declare.

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