**STDUY ON THE IMPACT OF ANILINE ON ANTIOXIDANT ENZYMES, BIOCHEMICAL CONTENTS AND HORMONAL ANALYSIS ON TESTIS OF MALE ALBINO RAT**

**ABSTRACT**

Aniline is an aromatic amine which is used very often in many fields of everyday use. It has increased its attention after the use of aniline as a byproduct in the synthesis of many chemicals. The production of paints, medicines, dyes, insecticides, and herbicides all make extensive use of anilines. Since it is used so widely, it can easily come into human contact and more studies on the effects of aniline are of utmost importance. Our study shows that aniline is responsible for showing various changes in treated rat such as reduction in body weight, organ weight, concentration of protein, DNA and concentration of hormones i.e. LH, FSH and Testosterone as compared to control animals. It is also causing oxidative stress because of which antioxidant enzymes are reducing and lipid peroxidation is increasing. Thus, our study highlights the detrimental effects of aniline in the testis and how aniline is affecting the overall health and well-being of the animal.

***Keywords*-** Aniline, lipid peroxidation, superoxide dismutase, catalase, LH, FSH, Testosterone.

**1. INTRODUCTION**

Aniline is an extensively produced industrial compounds worldwide. With the chemical formula C6H7N or C6H5NH2, the classic main aromatic amine is this one. It is made up of an amino group joined to a benzine ring. Benzene-amine, phenyl-amine, and amino-benzene are all synonyms for aniline. According to Liang et al. (2005) and Mujahid et al. (2010), aniline is a significant class of aromatic amine pollutant that poses a major threat to the environment. The class of aromatic amines includes aniline as a parent chemical. It is an important compound of many different products. It’s derivatives and by products are in the hundreds. Among the most widely utilized aniline derivatives are 1-naphthylamine (1-NPA), 4-chloroaniline (4-CA) 4,4'-methylenedianiline (4,4'-MDAand 3,4-dichloroaniline (3,4-DCA), Numerous paints, medicines, dyes, cosmetics, herbicides, rubber, and adhesives have all been made using aniline and its derivatives as intermediates or precious (Lewis, 2007; Mattarozzi et al., 2013; Saleh et al., 2016; Wang et al., 2016). In a number of nations, including the United States and European Union, a number of anilines are recognized as high quantity manufacturing compounds (European Commission, 2006; Di Girolamo et al., 2009; USEPA, 2009; Sihtmae et al., 2010). In 2016, the amount of aniline produced was 5.6 million tons (Wang et al., 2016).

Mammals easily convert aniline into a number of metabolites, such as 2-aminophenol, 4-aminophenol, and phenylhydroxylamine (Goodman et al., 1984). In humans, acute exposure to aniline can cause symptoms such headaches, nausea, vomiting, disorientation, vertigo, weakness, numbness, drowsiness, cyanosis from methemoglobinemia, and even coma (Gosselin et al., 1976).

Symptoms of long-term exposure include skin lesions, decreased body weight, and appetite loss (Weiss, 1986). Aniline exposure causes methemoglobinemia, hemolysis, and hemolytic anemia, according to toxicological research done on experimental animals (DiGirolamo, 2009). According to research by Ramteke (2021), aniline impairs renal function and metabolism by causing a variety of glomerular histology and subcellular structural alterations. Depending on the dosage concentration and amount of time after administration, aniline might induce renal injury. High aniline dosages were shown to affect renal function.

Very little is known about how aniline affects the male reproductive system, despite mounting evidence that it and its derivatives may disrupt biological processes. The purpose of this study is to determine how aniline affects the levels of hormones in the male albino rat's blood serum, biochemical components in the testis, and antioxidant enzymes.

**2. MATERIALS AND METHODS**

**2.1 Experimental Animal**

The investigation involved the selection of 24 male albino rats (Rattus norvegicus), weighing between 200 and 220 grams, under a 12-hour light/dark cycle at a regulated temperature of 25±2ºC.Each rat was housed in its own cage at the conditioned animal house of the Zoology department of RTM Nagpur University in Nagpur, India. Before the study started, the animals were acclimated for 14 days. the Institutional Animal Ethics Committee of RTM Nagpur University, Nagpur, authorized the experimental procedure (Register number 478/01/a CPCSEA). The rats were sacrificed at the conclusion of the procedure, and testicles were taken out and their weight was measured. Cardiac punctures were used to obtain blood samples for hormonal analysis.

**2.2 Treatments**

There were six rats in each of the four groups that were randomly selected. Group I was given normal saline water as a control, while Groups II, III, and IV were given oral dosage of aniline (Sigma Chemical Company, USA) in their drinking water for 20 days at doses of 30 mg/kg body weight (bw)/day, 60 mg/kg bw/day, and 90 mg/kg bw/day, respectively

**2.3** **Hormonal analysis**

2 ml blood sample was withdrawn through cardiac puncture using a disposable syringe and collected in a non-EDTA tube. The serum quantity of, follicle-stimulating hormone (FSH) luteinizing hormone (LH) and testosterone (T) were assessed using an ELISA kit.

**2.4 Anti-Oxidant Enzyme**

**a) Lipid peroxidation**

The method of Devasagayam and Tarachand (1987) was used for estimating lipid peroxidation (LPO) which involves quantifying the formation of malondialdehyde (MDA), a reactive aldehyde produced during the peroxidation of lipids. In this assay, tissue samples were homogenized in a Tris-HCl buffer with pH of 7.4, and the solution of mixture included 1 mL of a 0.15 mol/L Tris-HCl buffer. The MDA content is was then quantified spectrophotometrically, and the outcomes are given as nanomoles of MDA formed per milligram protein.

**b)** **Catalase**

The catalse activity was determinated as described by Aebi (1984) method. A tissue sample was mixed with a known concentration of hydrogen peroxide (H₂O₂) in a buffer solution. The activity was calculated by measuring the rate of the decrease in absorbance, which correlates to the concentration of hydrogen peroxide consumed.

**c) Superoxide dismutase**

With the help of an assay based on the decrease of nitro blue tetrazolium in accordance with the Total Superoxide Dismutase assay kit the superoxide dismutase activity was ascertained. The quantity of enzyme needed for 50% inhibition of nitro blue tetrazolium decrease at 560 nm at the temperature of 25°C was defined as one unit of enzyme activity. One milligram of GSH and one milligram of 1-chloro-2, 4-dinitrobenzene was used as the substrate in the spectrophotometric technique to measure the GST activity. One unit of glutathione S-transferase activity is the amount of enzyme that catalyzes the conjugation of 1 μmol 1-chloro-2, 4-dinitrobenzene and GSH at 37°C per minute.

**2.5 Biochemical estimation:**

**a) Protein estimation**

Protein concentration was quantified using the Lowry method (Lowry et al., 1951). Tissue samples and protein standards (bovine serum albumin, BSA) were diluted in appropriate buffers. To each sample, an equal volume of alkaline copper reagent (containing 2% CuSO₄, 0.1% Na₂CO₃, and 0.1 N NaOH) was added, and to enable the development of the protein-copper complex, the solutions were incubated for ten minutes at room temperature. Following incubation, Folin-Ciocalteu phenol reagent (diluted 1:1 with distilled water) was put in every tube, and the reaction was allowed to develop for a duration of 30 mins. Absorbance was noted at 750 nm with the help of a spectrophotometer.

**b) DNA estimation**

DNA estimation was performed using the Diphenylamine (DPA) method, a colorimetric assay based on the reaction between DNA and diphenylamine in an acidic environment. The DPA reagent reacts with deoxyribose, a component of DNA, forming a blue-colored complex that can be quantified spectrophotometrically. The intensity of the blue color correlates directly with DNA concentration, and absorbance was noted at 600 nm to determine the DNA concentration.

**2.6 Statistical Analysis**

The body weight and testis data of the control and aniline treated animal were statistically analysed using student’s t test to determine the significant difference between the aniline treated and control groups.

**3. RESULTS AND DISCUSSION**

**3.1 Morphological observation**

The control rats have a normal appearance and seem healthy. No appreciable morphological alterations were observed in the rat’s given aniline at low doses (30 mg/kg body weight), medium doses (60 mg/kg body weight), and high doses (90 mg/kg body weight) throughout a 20day period.

**3.2 Body weight**

The body mass of the control rats in this study increased from 210±0.57 to 213.83±1.37 gm after 20 days of providing them with a regular meal and normal saline water.
The rats that received doses of 30 mg/kg, 60 mg/kg, and 90 mg/kg of aniline showed a drop in body weight from the beginning to the end. The animal that received a high dose (90 mg/kg body weight) weighed 210.6±1.64 gm at the beginning and final 206.6±1.72 gm at the end of the treatment.

**Fig.1: Body weight of control and different doses of aniline treated rats for 20 days duration**.

**3.3 Testis weight**

The weight of the control group left and right testis was found to be 1.91±0.005 and 1.86±0.15 respectively. But in case of the aniline treated group, the weight of both testes decreased in a manner that was dependent on the dosage of aniline. The weight of the 30mg/kg body weight aniline treated left and right testis was found to be 1.8±0.022\*\*, 1.77±0.006\*\* and 1.55±0.006\*\*\*, 1.49±0.077\*\*\* respectively.

The weight of the 60mg/kg body weight treated aniline was 1.66±0.022\*\*\* and 1.64±0.006\*\*\*. In the case of the 90mg/kg body weight aniline treated animal, the testis weight was 1.55±0.006\*\*\* and 1.49±0.077\*\*\*. Thus, the weight of the testis was found to decline in a dose dependent manner.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.2: Organ weight of control different doses of aniline treated rat testis for 20 days duration.**

* 1. **Anti -oxidant enzymes**

**Super-oxide Dismutase**

Total Superoxide Dismutase concentration in control group testis of albino rat for 20 days duration was found to be 7.53±0.063 (U/mg). In the 30 mg/kg body weight aniline treated group, it was found to be 6.22±0.092\*\*\*(U/mg). In the 60 mg/kg body weight and 90mg/kg body weight it was found to be 6.15±0.022\*\*\*(U/mg) and 5.37±0.127\*\*\*(U/mg) respectively. Thus, super oxide dismutase is decreasing in a dose dependent manner in the testis.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.3: SOD concentration of control different doses of aniline treated rat testis for 20 days duration**

**Catalase**

Total Catalase concentration in control group testis of albino rat for 20 days duration was found to be 67.33± 0.07 (U/mg). In the 30 mg/kg body weight aniline treated group, it was found to be 66.96± 0.26 (U/mg). In the 60 mg/kg body weight and 90mg/kg body weight it was found to be 50.78±0.34\*\*\*(U/mg) and 38.67± 0.17\*\*\* (U/mg) respectively. Thus, catalase enzyme was found to decline in a dose dependent manner in the testis.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.4: Catalase concentration of control and different doses of aniline treated rat testis for 20 days duration**

 **Lipid Peroxide**

Total LPO concentration incontrol group testis of albino rat for 20 days duration was found to be 2.8± 0.06 (n moles MAD/mg protein). In the 30 mg/kg body weight aniline treated group, it was found to be 3.18± 0.106 (n moles MAD/mg protein). In the 60 mg/kg body weight and 90mg/kg body weight t was found to be 3.78± 0.07\*\*\*(n moles MAD/mg protein) and 4.25± 0.13\*\*\*(n moles MAD/mg protein) respectively. Thus, lipid production was found to increase in a dose dependent manner in the testis of the animal.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.5: Lipid peroxidation concentration of control and different doses of aniline treated rat testis for 20 days duration**

* 1. **Biochemical Contents**

**Protein**

Total protein in control group testis of albino rat for 20 days duration was found to be 1.40±0.01 (mg/gm). In the 30 mg/kg body weight aniline treated group, it was found to be 1.29±0.03\* mg/gm. In the 60 mg/kg body weight and 90mg/kg body weight it was found to be 1.22±0.04\* mg/gm and 0.98±0.05\*\*\* mg/gm. respectively. Thus, it is evident that, as the dosage of aniline increases, the protein concentration in the testis decreases.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.6: Protein concentration of control and different doses of aniline treated rat testis for 20 days duration**

 **DNA**

Total DNA in control group testis of albino rat for 20 days duration was found to be 0.417±0.127 mg/gm. In the 30 mg/kg body weight aniline treated group, it was found to be 0.317±0.003\*\*mg/gm. In the 60 mg/kg BW and 90mg/kg body weight it was found to be 0.279±0.003\*\*\*mg/gm and 0.236±0.017\*\*\*mg/gm. Respectively. Thus, it is evident that, as the aniline dosage increases, the DNA concentration decreases in the testis of the animal.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.7: DNA Concentration of control and different doses of aniline treated rat testis for 20 days duration**

**3.6 Hormonal Analysis**

 **Serum** **Luteinizing Hormone**

Total LH in control group testis of albino rat for 20 days duration was found to be 0.103±0.0004miU/ml. In the 30 mg/kg body weight aniline treated group, it was found to be 0.100±0.0004\*\* miU/ml. In the 60 mg/kg body

weight and 90mg/kg body weight it was found to be 0.097±0.0004\*\*\* /ml. and 0.092±0.0002\*\*\*miU/ml respectively. Therefore, when the dose of aniline is increasing, the total LH is decreasing in the blood serum of the animal.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.8: LH concentration of control and different doses of aniline treated rat serum for 20 days duration**

**Serum Follicle Stimulating Hormone**

Total FSH in control group testis of albino rat for 20 days duration was found to be 0.302±0.0004 miU/ml. In the 30 mg/kg body weight aniline treated group, it was found to be 0.286±0.002 miU/ml. In the 60 mg/kg body weight and 90mg/kg body weight it was found to be 0.272±0.002\*\*\*miU /ml and 0.264±0.001\*\*\* miU/ml respectively. Therefore, when the dose of aniline is increasing, the total FSH is decreasing in the blood serum of the animal

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*,p<0.0001\*\*\*

**Fig.9: FSH concentration of control and different doses of aniline treated rat serum for 20 days duration**

**Testosterone Hormone**

Total Testosterone in control group testis of albino rat for 20 days duration was found to be 385.52±0.767(ng/dl). In the 30 mg/kg body weight aniline treated group, it was found to be 326.35±2.97\*\* (ng/dl). In the 60 mg/kg body weight and 90mg/kg body weight it was found to be 313.5±0.341\*\*\* (ng/dl) and 300.5±0.22\*\*\*(ng/dl) respectively.

Therefore, when the dose of aniline is increasing, the total Testosterone is decreasing in the blood serum of the animal.

Values expressed as mean± SEM (n=6 for each group), p<0.05\*, p<0.001\*\*, p<0.0001\*\*\*

**Fig.10: Testosterone concentration of control and Aniline treated rats for 20 days duration**

**DISCUSSION**

Several studies have indicated that exposure to aniline can lead to reduced weight gain or weight loss in rats. A study by **(**Pauluhn, 2004)investigated the sub-chronic effects of aniline on rats and found that it could cause a decline in body mass, likely because of liver damage and impaired metabolism. (Khan, 1993)observed that rats chronically exposed to aniline via inhalation developed signs of weight loss, possibly due to the compound’s toxic effects on multiple organ systems. In a study by (Hejtmancik, 2002)it was seen that body weight decreased after administration of aniline. These results are in line with our study. Aniline has the potential to adversely affect rat weight, particularly through mechanisms related to liver and kidney damage, metabolic disruption, and haematological changes. Multiple animal studies have demonstrated that aniline exposure results in a reduction in testis weight, which is often a sign of organ atrophy. When aminophenylnorharman (a derivate of aniline)at a dosage of 90 mg/kg was given to albino rats, atrophy in testicular area was noted by (Totsuka, 2001). Similar results were observed by (Zhang, 2009) were testis weight decreased significantly with the increased 3,4-Dichloroaniline concentration. Similar results were observed in our study. This decrease in weight can be linked to a variety of mechanisms including direct toxic effects on testicular cells and alterations in hormonal regulation.

In the present study, exposure to aniline has been shown to induce changes in protein levels and expression patterns in the testes, suggesting that aniline interferes with the normal functioning of testicular cells. The primary effects are linked to oxidative stress. (Rodriguez, 2006) showed that similar effects on protein levels were seen in the testis of rats when it was injected with a chemical pesticide Parathion. Similar results in the rat testis were seen when chloroquine was given to the animal at the dose of 2mg/kg body weight. (Jiwantare, 2021)

The results revealed that the total level of DNA concentration is also decreasing as the dose of aniline is increased. According to (Eissa, 2012) DNA concentration changes may be caused from: (i) straight DNA breaking, (ii) duplication on a dented DNA plan, (iii) reserve of DNA synthesis and other apparatuses like topoisomerase II inhibition. This may be the causes of reduction in total DNA amount in the treated rats. One study by (Totsuka, 2001) showed that aniline exposure resulted in a significant reduction in testosterone levels, possibly due to its toxic effects on the Leydig cells in the testes, that are accountable for testosterone manufacture. This explains the reduction in testosterone in our study. Rats exposed to aniline found altered levels of LH and FSH. While testosterone levels were reduced, LH levels were also significantly reduced in aniline-treated animals, indicating a possible disruption in the feedback loop regulating hormone production in the testes. This could be due to aniline’s direct toxic effects on the testes or its interference with the hypothalamic-pituitary-gonadal axis.

A study by (Eissa *et al*., 2012) found that exposure to aniline led to a significant increase in ROS production in the testis, contributing to oxidative damage. Antioxidants such as catalase, superoxide dismutase (SOD), and glutathione (GSH) are important for maintaining cellular redox balance. In a study by (Jiwantare, 2021) superoxide dismutase (SOD), catalase was seen to reduce when chloroquine was given to rats at the dose of 2mg/kg bw for a duration of 30 and 60 days. These results are similar to our study, thus proving that aniline causes the production of reactive oxygen species.

**CONCLUSION**

The animal loses weight when exposed to aniline. Additionally, it results in a reduction in the levels of hormones such as testosterone, FSH, and LH, which leads to a hormonal. Therefore, aniline might have played a role in male’s inability to reproduce. The results of this study will help identify derivatives of potential toxicological concern and will motivate more researchers to investigate the impact of aniline derivatives on endocrine disruption.

**ETHICAL APPROVAL**

Animal Ethic committee approval has been collected and preserved by the author(s)

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

We hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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