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Histomorphometric Evidences of the Effects of *Allium Sativum* on Cyclophosphamide Induced Testicular Toxicity in Adult Wistar Rat

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Abstract

Cyclophosphamide (CYP) is a widely used anticancer drug that can also induce testicular toxicity, hence male factor infertility. This adverse effect of CYP can be ameliorated by the antioxidant effect of garlic oil. This research work investigated the effects of Garlic oil (GO) on CYP-induced testicular toxicity in Adult Male Wistar rats. Thirty-six adult male Wistar rats were divided into 12 groups, each containing three (3) rats. Groups 1 and 2 were treated for 21 and 28 days respectively using 2mg/kg/BW of Normal saline. Groups 3 and 4 were treated for 21 and 28 days respectively using 90mg/kg/day of GO. Groups 5 and 6 were treated for 21 and 28days respectively with CYP (50mg/kg/wk). Groups 7 and 8 were treated for 21 and 28 days respectively with both GO (90/kg/day) and CYP (50/kg/wk). Groups 9 and 10 were treated for 21 days and 28 days respectively with GO (60mg/kg/day) and CYP (50mg/kg/wk). Groups 11 and 12 were treated for 21 and 28 days respectively using GO (30mg/kg/day) and CYP (50mg/kg/wk). The animals were sacrificed 24 hours after the last treatment by decapitation. The testes were harvested and processed for H&E staining. The values were presented as means of standard error of means (SAM). Statistical analysis was carried out using one-way analysis of variance (ANOVA). The result showed significant ($p \leq 0.05$) decrease in the final body weight of rats treated with only CYP. Histology findings in CYP alone groups (5 and 6) were that of seminiferous tubular atrophy and differential germ cell disorganization. The result from this study also revealed a significant ($p \leq 0.05$) decrease in normal sperm morphology and significant increase in abnormal morphology in both acute and chronic administration of CYP. This study has generated data showing that CYP disrupts redox balance in testicular tissues and thereby impair testicular histology. Importantly, it has been demonstrated that GO ameliorates these harmful effects of CP on reproductive function.

Keywords: garlic oil, cyclophosphamide, histomorphology, testes

1. Introduction

Infertility is one of the reproductive health challenges with far reaching economic and psychological implications especially in an African society, with high premium on child-bearing. (Alkreathy, Z. A., Damanhouri, N., Ahmed, M., Slevin, S. S. & Ali, A. M., 2010) Male factor contributes about 30%-50% of all the cases of infertility. (Roozbeh N, Rostami S, & Abdi F., 2016) Here are several causes of male factor infertility which include; physical causes (blockage of the ejaculatory pathway, varicocele, testicular torsion), hormonal deficits, genetic factors, sexually transmitted infections (Chlamydia & Gonorrhea), environment factors (solvents, insecticides, adhesives, and radiation), life style (alcohol and smoking) and very importantly drugs (cyclophosphamide). (Krausz, C. & Riera-Escamilla, A., 2018; Lee, J. A. & Ramasamy, R., 2018; Lotti, F. & Maggi, M., 2018; Winters, B. R. & Walsh, T. J., 2013)

Cyclophosphamide (CYP) is an efficacious anticancer alkylating agent that is commonly used for the treatment

of several malignancies, as well as an immunosuppressive agent for organ transplantation, systemic lupus erythematosus, multiple sclerosis and other benign tumors. (Ayoka, O. A., Ojo, O. E., Imafidon, E. C., Ademoye, K. A. & Oladele, A. A., 2016) Despite its clinical uses, CYP is restrained because of its many adverse effects such as; hepatotoxicity, nephrotoxicity, cardiotoxicity and reproductive toxicity in both animal and human model. (Arena, A. C., Jorge., B., Silver, M. C & de Barros, A. L., 2018) There are many research works that have shown that CYP induces derangement in spermatogenesis and causes atrophy of seminiferous tubules and testosterone level depletion. (Kaur, F., Sangha, G. K & Bilaspuri, G. S., 1997) There are also reports on the CYP induced biochemical and histological alterations in the testis and epididymis of humans and rats. (Maremanda, K. P., Khan, S. & Jena, G., 2014) The CYP induced reproductive deficits could be triggered by the generation of reactive oxygen species (ROS) and lipid peroxidation as suggested by previous studies (Sulkowski, 1998).

The search for natural products that may preserve the anticancer drug efficacy while mitigating its side effects is the goal of many studies. Recently, the beneficial health efficacy of natural oil is emerging in the literature, and they appear attractive to consumers because they are amenable to daily diet. (Chima, A., Ekeleme-Egedigwea, Ademola, C. Famurewab, C., Ebuka, E., Chinedum, O., Eleazua, D. & Uchenna, O., 2019)

Garlic (*Allium sativum*) is a common spicy flavoring agent and medicinal herb. It is used since ancient times for prevention and treatment of various ailments. Literature has revealed many pharmacological properties of garlic and its derivative compounds by epidemiological studies and animal experiments. (Alkreathy, Z. A., Damanhouri, N., Ahmed, M., Slevin, S. S. & Ali, A. M., 2010) Studies have shown that garlic oil has the ability to lower blood pressure and cholesterol levels thereby reducing the risk of atherosclerosis and hypertensive heart disease. (Aqel, M. B., Gharaibah, M. N. & Salhab, A. S., 1991) Allium sativum is an antioxidant and a detoxifying agent, which scavenges the reactive oxygen species (ROS), improves the cellular antioxidant enzymes, (such as glutathione peroxidase, superoxide dismutase, catalase, etc.) thereby protecting the body against oxidative stress induced disease. (Banerjee, S. K., Dinda, A. K., Manchanda, S. C. & Maulik, S. K., (2002)

2. Materials and Methods

2.1 Chemical

Cyclophosphamide (Shandong Octagon Chemicals LTD, China) and marketed by Vical Pharmacy Makurdi. Assay kits for antioxidant enzyme and Thiobarbituric acid (TBA) produced by MP biomedicals and marketed by blessings Laboratories Ltd Ibadan.

2.2 Animal

Thirty-six (36) male Wistar rats age ranging from 2-5 months (Sengupta, P., 2013) and weighing 160–200g, were procured from Animal House, college of health science, Benue State University Makurdi. Animals were housed in the house as above, in a room at 25 ± 2 °C with a lighting schedule of 12h light and 12h dark. Rats were fed with commercial pelleted rat growers mash diet (Vital Feed, Jos, Nigeria) and water.

2.3 Garlic Oil

Refined GO gel produced by Adva Care Pharm was procured from Vical Pharmacy Makurdi.

2.4 Experimental Design

This study involved 12 groups of male Wistar rats to evaluate the effects of garlic oil and cyclophosphamide over different durations. Groups 1 and 2 served as controls, receiving normal saline for 21 and 28 days, respectively. Groups 3 and 4 were administered garlic oil (90 mg/kg) for 21 and 28 days. Groups 5 and 6 received cyclophosphamide (50 mg/kg) weekly for 21 and 28 days. Groups 7 and 8 received a combination of garlic oil (90 mg/kg/day) and cyclophosphamide weekly for 21 and 28 days, while groups 9 and 10 received garlic oil at a lower dose (60 mg/kg/day) combined with cyclophosphamide. Finally, groups 11 and 12 were given garlic oil (30 mg/kg/day) with cyclophosphamide over the same time periods. The study explored the protective potential of garlic oil against cyclophosphamide-induced effects.

2.5 Animal Sacrifice

The animals were sacrificed by decapitation and the testes carefully excised immediately and rinsed in ice-cold physiological saline, and the weight determined.

Testes tissues were homogenized in 0.1 M phosphate buffered saline (1:5 w/v, pH 6.4). A portion of testis was preserved in 10% formalin for histological analysis.

2.6 Ethical Considerations

Ethical permission was secured from the Ethical Committee, College of Health Science, Benue State University Makurdi (CHS REC NO CREC/003).

2.7 Histological Study

Testis samples were fixed in 10% buffered formalin for 48h and dehydrated in ethanol and embedded in paraffin. Sections were cut by rotary microtome and stained with hematoxylin and eosin (H and E) for microscopic histopathological changes. The slides were viewed and examined with compound light microscope. (Saalu, L. C, 2016)

2.8 Statistical Analysis

The values were presented as standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Tukey's Post hoc test. The value of $p \leq 0.05$ was regarded as statistically significant.

3. Results

3.1 Gross Anatomical Parameters

As shown in Table 1, there was significant ($p \leq 0.05$) difference between the initial body weight, final body weight and the body weight difference of experimental and control rats.

Table 1. Effect of GO and CYP on the gross anatomical parameters of Wistar rat

Groups	Treatment	Initial Body Wt. (g)	Final Body Wt. (g)	Body Wt. Differences
1	Acute Normal Control	195.50±0.70	82.45±116.6	-30.10
2	Chronic Normal Control	197.25±15.20	168.50±3.53	-28.75
3	Acute GO	205.50±19.00	189.0±0.00	-30.00
4	Chronic GO	00±00	00±00	00.00
5	Acute CYP	220.00±2.82	197.35±17.88	-22.65
6	Chronic CYP	212.50±10.6	207.50±10.60	-5.00
7	Acute High GO + CYP	187.00±8.48	160.75±30.61	-26.3
8	Chronic High GO + CYP	183.0±0.00	192.0±0.00	9.00
9	Acute Medium GO + CYP	195.75±20.15	187.95±21.9	-7.8
10	Chronic Medium GO + CYP	176.00±2.82	159.7±0.70	-16.3
11	Acute Low GO + CYP	00±00	00±00	0.00
12	Chronic Low GO + CYP	00±00	00±00	0.00

3.2 The Effect of GO and CYP on Abnormal Sperm Morphology (Round Head, Bent Mid-Piece, Rudimentary Tail)

As shown in Table 1 and supported by plates 1,5 and 10, there was a significant ($p \leq 0.05$) increase in sperm with round Head, Bent Mid-piece and rudimentary tail in group 5 (positive control) when compared to groups 1 (negative control). The groups that were post-treated with GO had a significant ($p \leq 0.05$) decrease in sperm with round Head, Bent Mid-piece and rudimentary tail when compared to the groups that were treated with CYP-alone (5).

The groups treated with higher and medium dose of GO recorded sperms with more ($p \leq 0.05$) rudimentary tail when compared when compared to the low-dosed groups. Sperm with Bent Mid-piece was significantly ($p \leq 0.05$) higher in chronic of low and medium dosed groups when compared to the acute counterparts.

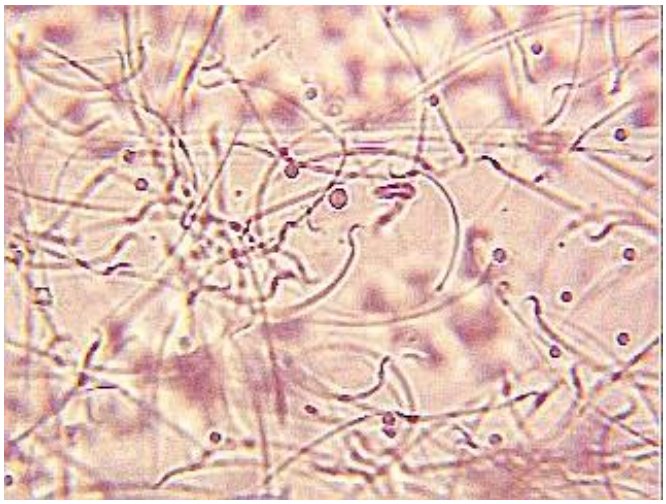

Plate 1. Photomicrograph of sperm from group 1 (acute normal control). Treatment: 2ml/kg body weight of NS. Magnification: x10

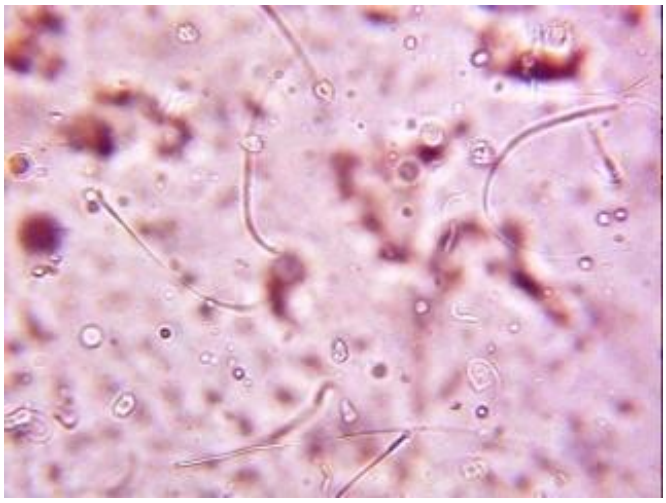

Plate 5. Photomicrograph of sperm from group 5 (Acute Cyclophosphamide). Treatment: 50mg/kg body weight of Cyclophosphamide. Magnification: x10

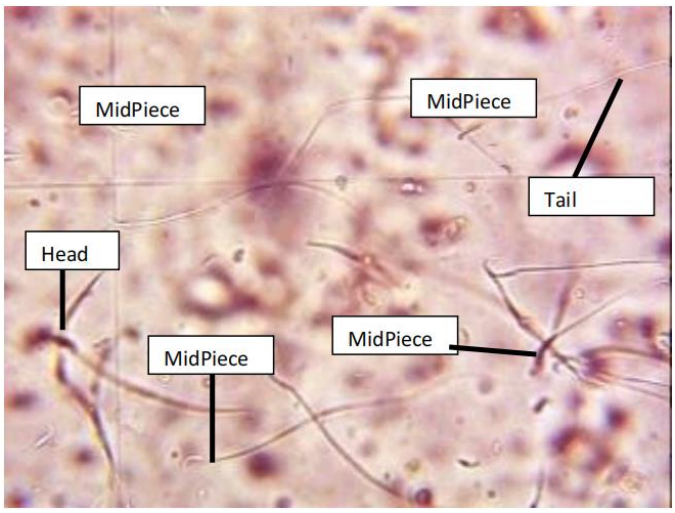

Plate 10. Photomicrograph of sperm from group 6 (Chronic Medium G. oil + Cyclophosphamide). Treatment: 30mg/kg and 50mg/kg body weight of garlic oil extract and Cyclophosphamide respectively. Magnification: x10

3.3 Histological Profile

Histological findings of the testis in group 1-4 showed normal histological features with numerous spermatozoa radiating towards the lumen, the Leydig cells were also intact. Spermatid retention was found in the seminiferous tubules (Plate 13-16).

Group 5 and 6 had abnormal morphology of seminiferous tubules, spermatid retention and/or tubular atrophy and diffuse germ cell disorganization were also observed. The testicular profile showed absence of interstitial space, degeneration of testicular architecture and necrosis. Interstitial tissues, several maturing spermatogenic cells in seminiferous tubules of testis appeared. In some cells the nuclear membrane had been ruptured and was accompanied by fragmentation of nucleus (karyorrhexis) (Plate 17-18).

The profile of groups 7-12 showed spermatogonia cells with darkly stained nuclei and destroyed sperm cells, majority of seminiferous tubules were shrunken and had a wavy outline. The basement membranes were thickened and hyalinised. Debris of shredded cells occupied most of the lumen of the seminiferous tubules. One could say they were similar to groups 5 and 6 but can't be compared to groups 1 to 4 as shown in Plate 19-24.

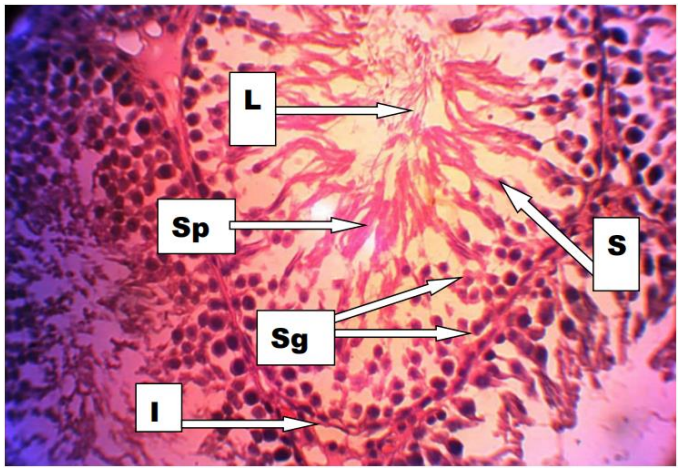

Plate 13. Photomicrograph of the testes of rat from group 1 **(**acute normal control). Treatment: 2ml/kg body weight of NS. L: Lumen, Sp: Spermatozoa, Sg: Spermatogonia, I: Interstitial cells, S: Sertoli cells, Stain: H&E; Magnification: x40

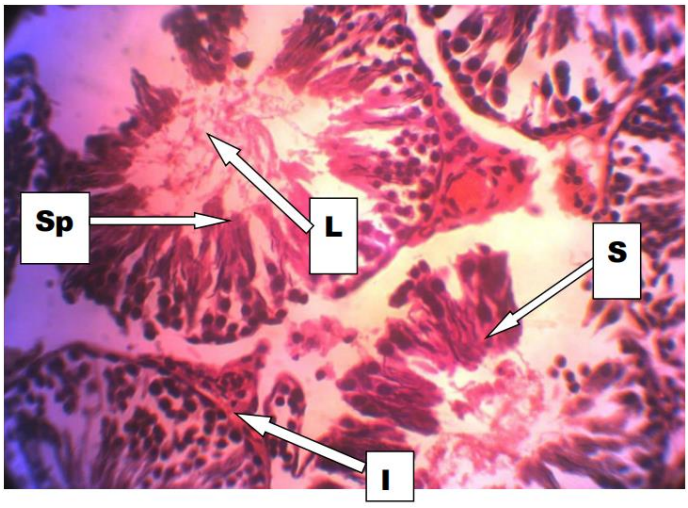

Plate 15. Photomicrograph of the testes of rat from group 3 **(**Acute Garlic oil). Treatment: 90mg/kg body weight of Garlic oil extract. L: Lumen, Sp: Spermatozoa, Sg: Spermatogonia, I: Interstitial cells, S: Sertoli cells, Stain: H&E; Magnification: x40

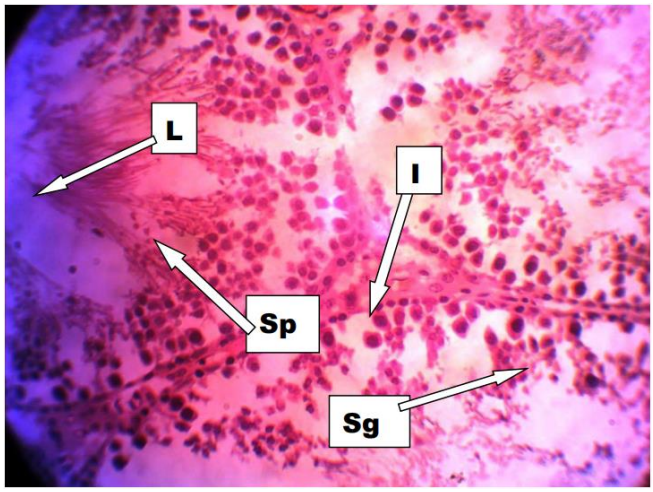

Plate 17. Photomicrograph of the testes of rat from group 5 **(**Acute Cyclophosphamide). Treatment: 50mg/kg body weight of Cyclophosphamide. L: Lumen, Sp: Spermatozoa, Sg: Spermatogonia, I: Interstitial cells, S: Sertoli cells, Stain: H&E; Magnification: x40

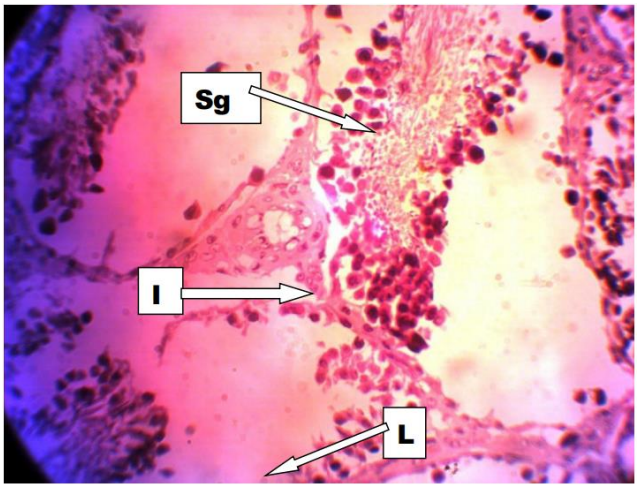

Plate 18. Photomicrograph of the testes of rat from group 6 **(**Chronic Cyclophosphamide). Treatment: 50mg/kg body weight of Cyclophosphamide. Stain: H&E; Magnification: x40

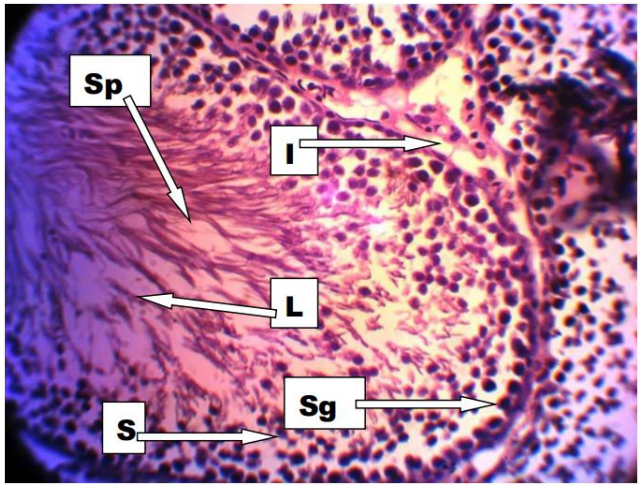

Plate 20. Photomicrograph of the testes of rat from group 6 **(**Chronic High G. oil+Cyclophosphamide). Treatment: 90mg/kg and 50mg/kg body weight of garlic oil extract and Cyclophosphamide respectively. Stain: H&E; Magnification: x40

4. Discussion

Gonadotoxicity is one of the most prominent and most frequently encountered side effects associated with prolonged use of CYP and there is an urgent need to discover therapeutic means to mitigate or preserve the reproductive viability of cancer patients exposed to CYP treatment.

In the present study, we investigated the histomorphological evidence of the effects of Allium sativum (garlic oil) on Cyclophosphamide induced testicular toxicity in adult Wistar rat. It was aimed at investigating the Gonado-protective effects of GO against CYP-induced Gonadotoxicity in male rats by assessing the impact of the treatment on histomorphological alterations.

The result from this study revealed a significant ($p \leq 0.05$) decrease in normal sperm morphology and significant increase in abnormal morphology in both acute and chronic administration of CYP. However, this effect was seen more in the group with chronic exposure of CYP. This result is in keeping with other previous studies (Elangovan, N., Chiou, W.F. & Tzeng, S., 2006; Zhang, C. L., Zeng, T., Zhao, O. L., Yu, L. H., Zhu, Z. P. & Xie, K. Q., 2012).

In this study also, the histomorphological examination of the groups of rats treated with CYP revealed severe arrest of spermatic tissue and necrosis of cells involved in spermatogenesis and testosterone production. The results also showed cells with irregular and dense nuclei in the lumina of seminiferous tubules with incomplete maturation of spermatogonia and vast interstitial space tubules in the CYP-treated groups. This result is in agreement with other similar studies. (Saber A., Somaya Y., Shalaby. & Rawan H., 2017) Cyclophosphamide is a DNA alkylating agent that prevents DNA replication during cell division, leading to cell cycle arrest at the S phase and induction of apoptosis in telencephalon embryonic neural progenitor cells at 6–12h after administration. (Twilley, D., Kishore, N., Meyer, D., Moodley, I., Kumar, V., & Lall, N., 2017) Following administration of CYP to rats, atrophy of seminiferous tubules with interstitial vacuolation may decrease sperm viability and motility, as reported in multiple studies. (Zhao, H. et al., 2015; Ayoka, O. A., Ojo, O. E., Imafidon, E. C., Ademoye, K. A. & Oladele, A. A., 2016). CYP is shown to increase the absorption of testicular haemoglobin, which is a hallmark of oedema and haemorrhage, as indicated by histomorphological analyses. (Besikcioglu, 2019) These effects reflected associations of CYP with proteins containing sulfhydryl groups. Furthermore, excessive reactive oxygen species (ROS) generation likely damages blood vessels directly. (Besikcioglu, 2019) Reactive oxygen species and oxidative stress mediated inflammation has been implicated in the pathophysiology of CYP -induced toxicity including testicular damages. (Vernet, P., Aitken, R. & Drevet, J., 2004) Oxidative stress plays a critical role in the pathogenesis of reproductive disorders and the etiology of defective sperm function. (Kothari, S., Thompson, A., Agarwal, A & Plessis, S. S., 2010)

5. Conclusion

Allium sativum has been demonstrated in this study to possess antioxidant properties by mitigating the effect of CYP induced testicular toxicity in both acute and chronic phase in adult Wistar rat. It has also been shown that both CYP and high dose Allium sativum are detrimental to the testicular architecture and may hence affect male fertility. Further studies should be carried out in higher animals like human beings to explore the option of using Allium sativum concomitantly with anticancer agents like CYP considering its availability and affordability.

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