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CO-ADMINISTRATION OF ZINC AND ASCORBATE ATTENUATES GERM CELL mRNA ANDROGEN RECEPTOR IN VALORPHIN INDUCED TESTICULAR-TOXICITY IN WISTAR RATS

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ABSTRACT

Introduction: Opioids are substances that act on opioid receptors to produce morphine-like effects. Medically, they are primarily used for pain relief and reports have shown that Valorphin causes degenerative changes to the testes. This will in turn reduce male fertility. Thus, this work aims to investigate the effects of the pain relief drug (Valorphin) on the testes and the effect of co-administration of Zinc and Ascorbate on Adult Male Wistar Rats.

Methods: Twenty-five (25) Wistar rats weighing between 150-180 g were used and randomly assigned into five groups designated as A, B, C, D, E (n = 5). Group A was designed as a control group which received normal saline while Group B received 3 mg/kg body weight of Valorphin only, Group C received 3 mg/kg of Valorphin and 150 mg/kg of Vitamin C, Group D received 3 mg/kg of Valorphin and 7 mg/kg of Zinc only, while Group E received 3 mg/kg of Valorphin 150 mg/kg of Vitamin C and 7 mg/kg of Zinc.

Results: Serum testosterone concentration, percentage motile sperm, superoxide dismutase (SOD), and glutathione peroxidase (Gpx) concentrations were significantly reduced in the Valorphin-only treated group, while the androgen receptor (AR) gene was also down-regulated in this group. Significant

increases in all these parameters (testosterone, motile sperm, SOD, Gpx, and AR gene) were observed in the groups where Valorphin was co-treated with zinc and vitamin C. Histopathologic results showed degenerative changes in the group administered with Valorphin only, and their lumen was devoid of sperm cells.

Conclusion: Valorphin has been shown to have a deleterious effect on the testes, primarily by its influence on the hypothalamic-hypophyseal-gonadal axis, while co-administration of zinc and vitamin C with valorphin significantly ameliorated this testicular toxicity.

Keywords: Reproductive toxicity, Valorphin, Fertility, Anti-oxidant, Gene-expression

INTRODUCTION

The evidence of fertility being the ability of a man to impregnate his wife and the couple having their children is a very important issue for the harmony of married couples. The inability to prove fertility with conception and the birth of a living healthy child is capable of causing disaffection in a family. In Africa, infertility prevalence rates range from 20-35%. In Nigeria, 10-30% of couples have problems with fertility. The male factor is responsible for about 40-50% of all infertility cases.¹

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Male fertility depends on the seemingly anatomical and physiologically matured male producing normal sperm and delivering them during copulation. Male fertility depends on the continuous production of sperm by the testes. This consists of a process of cellular events, called spermatogenesis. However, for the sperms to be delivered into the female vagina during sexual intercourse, the male must be capable of sustainable erection with eventual ejaculation of the sperms into the female vagina and further passage to the cervix, uterine lumen, and the fallopian tubes where the sperms meet the ova released from the ovary for fertilization.

One of the major risk factors for male infertility is reproductive toxicity from substances such as opioids. Opioids generally decrease not only luteinizing hormone (LH) but also testosterone and estradiol, which have effects on testicular function.^{2,3} Hypogonadism, infertility, reduced libido and erectile dysfunction have been linked to Opioid abuse in men.⁴

Reports have also shown an opioid-driven impairment of the hypothalamic function that can result in a loss of libido, impotence, and infertility.⁵ Akhigbe and Ajayi also showed the adverse effect of opioids on the testes and male fertility, as it affected the architecture and micro-histology of the testes.⁶ One of these opioids is Valorphin which is a naturally occurring, endogenous opioid that can also be synthesized. It works through the *mu* receptor just like most opioids and morphine.

Antioxidants such as Zinc and Vitamin C have proved to be useful remedies. Zinc is vital for male fertility, as low Zinc levels have been demonstrated in men with low testosterone and poor sperm quality.⁷ Equally L-ascorbic acid or vitamin C (also known as ascorbate) is a vitamin found in various foods and sold as a dietary supplement. Studies have shown that some nutritional therapies improve sperm counts and sperm motility. The purpose of this study is to examine the oxidative effects of Valorphin on the testes and the ameliorative actions of vitamin C and Zinc as antioxidants.

METHODS

Experimental Animals

A total number of twenty-five male (25) Wistar rats weighing between 150-180 g were used and the animals were randomly divided into five groups (A, B, C, D, E) with each group comprising five rats. Group A was designed as a control group which received normal saline while Group B received 3 mg/kg body weight of Valorphin only, Group C received 3 mg/kg of Valorphin and 150 mg/kg of Vitamin C, Group D received 3 mg/kg of Valorphin and 7 mg/kg of Zinc only while Group E received 3 mg/kg of Valorphin 150 mg/kg of Vitamin C and 7 mg/kg of Zinc. Drug administration was done orally using orogastric cannula for 28 days.

The compound of study

Valorphin, Ascorbic acid, and Zinc were purchased from Hefei TDJ Chemical Co. Ltd in China.

Sample collection and preparation

Twenty-five adult male Wistar rats were euthanized by cervical dislocation 12 hours after the last administration. The testes and caudal epididymis were excised following an abdominal incision and fixed in Bouin's fluid for histological analysis. The fixed tissues underwent processing and the histological slides were examined for possible changes.

Procedure for Testosterone Hormonal Analysis

Serum testosterone was measured in 10- μ L serum-testosterone assays. This was carried out with the use of the respective ELISA Kit. The binding sites of the testosterone antiserum coated to the wells of a microplate are in competition with an unknown amount of testosterone present in the sample and a known amount of testosterone conjugated to horseradish peroxidase. The microplate was rinsed four times after an hour of shaking incubation. The concentration of testosterone is demonstrated to be inversely related to the optical density measured following the addition of the substrate solution.

Procedure for Sperm Motility

A few locations on the caudal epididymis were cut with a scalpel blade before it was

removed and put in a Petri dish with a small amount of buffer. Blood vessels were avoided and the scalpel blade reaches into but not into the duct lumen. To prevent sperm exposure to air and promote dispersion within the buffer, the tubule segment was submerged in the buffer. The tissue was removed after the sperm had been allowed to diffuse into the liquid for 0.5 to 15 min. The sperm were then incubated until sufficiently dispersed for examination. A chamber was created by using an aliquot of the sperm suspension in a 60 x 15 mm polystyrene dish and overlaying it with a 22 x 22 mm cover-slip and the sperm velocity was observed under light microscope. The percentage of motile sperm was calculated by the number of motile sperm/total number of sperm x 100.

Reverse Transcriptase Polymerase Chain Reaction Analysis for Androgen Receptor

ThermoFisher Scientific's TRIzol Reagent was used to isolate RNA from the testis. cDNA was produced right away from purified, DNA-free RNA using the ProtoScript® First Strand cDNA Synthesis Kit from NEB. A 50 uL reaction mixture containing 10 uL of PCR buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl/1.5 mM MgCl₂), 2.5 uL of each deoxynucleotide triphosphate (10 mM), and 5 uL of forward and reverse (10 mM) primers was used to amplify total cDNA (5 uL, 10 ng). The following conditions were used for the amplification: 5 min of pre-denaturation at 94°C, 30 sec of denaturation at 94°C, 30 sec of annealing at 58°C, 30 sec of extension at 72°C, and 5 min at 72°C for 30 cycles. On a 5% Agarose gel, the amplicons produced during the PCR stage were resolved. An iPhone X camera was used to capture the in-gel expression bands (Noir effect). Gel image post-processing was carried out on a MacBook Pro iOS computer using the Keynote platform. GraphPad Prism (version 8.4.3.686) was used to create the bar chart displaying the relative expression of androgen receptor genes after performing the densitometric analysis using Image-J software (version 1.53n).

Histological Processing

Hematoxylin and eosin staining procedures were used for histological processing. The testes were first fixed in Bouin's solution, then dehydrated in progressively increasing concentrations of alcohol, cleared in xylene, infiltrated with molten paraffin wax, and embedded in molten paraffin wax to block. Rotary microtome was then used to cut the paraffin block containing the tissue into 4µm thick sections. The sections were then floated in a water bath set at 40°C, transferred to a glass slide, dried on a slide warmer, and stained using hematoxylin and eosin stain.

Statistical Analysis

The mean and standard error of the mean (S.E.M) of all the data were calculated. Comparison of means was made by one-way analysis of variance (ANOVA) using GraphPad Prism 5. Tukey's test was used to adjust for multiple comparisons and p-value < 0.05 was considered to be statistically significant.

RESULTS

Result from Figure 1 revealed serum testosterone concentration mean values after the administration of Valorphin, zinc, and ascorbate. High significant decrease in serum testosterone concentration was observed in Group B (**p<0.001) when compared with control Group A and group E rats. Furthermore, slight significant increase in serum testosterone concentration was also observed in Groups C and D (*p<0.05) when compared with Valorphin-only treated group B.

Mean values of Androgen Receptor gene expression among experimental groups has shown in Figure 2 revealed high significant down-regulation of Androgen Receptor gene in Valorphin only treated group B (**p<0.001) as compared to Groups A and E rats. There was no significant difference in Androgen Receptor gene expression in groups C and D when compared with control group A rats.

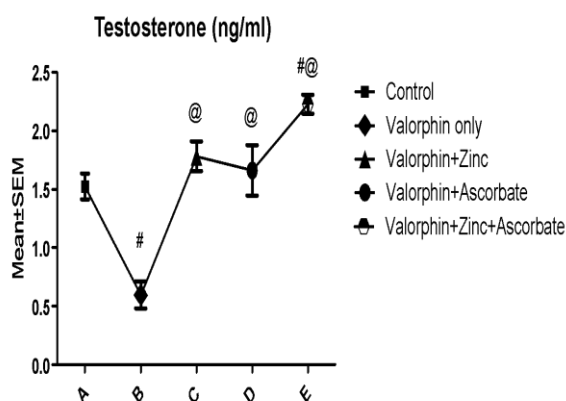


Figure 1: Showed comparison in Serum testosterone hormone Concentration among the groups after the administration of Valorphin, zinc, and ascorbate. (n=5). # = Comparison with Control Group A; @ = Comparison with Group B.

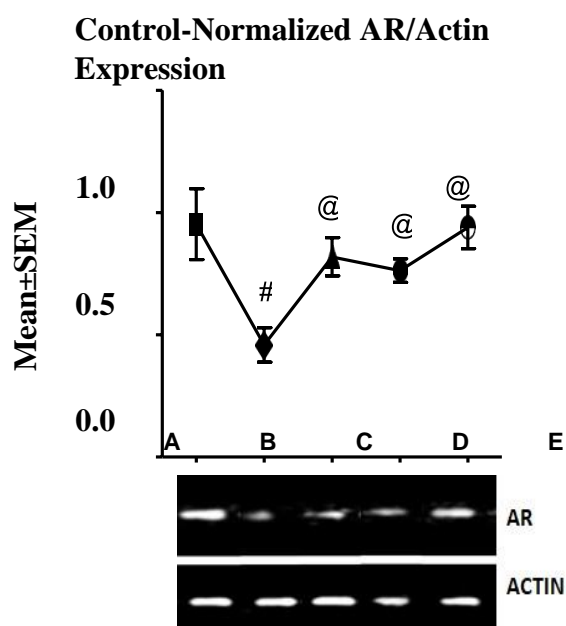


Figure 2: Showed comparison in Androgen Receptor Gene expression among the groups after the administration of Valorphin, zinc, and ascorbate, (n=5). # = Comparison with Control Group A; @ = Comparison with Group B.

Result from Figure 3, showed mean values for sperm motility after administration of Valorphin, zinc, and ascorbate. Concentration of motile spermatozoa was significantly decreased in Valorphin treated group B when compared with control group A ($***p<0.001$) while slight significant decrease in motile spermatozoa was observed in group B when compared with groups C, D and E ($*p<0.05$).

No significant difference in sperm motility was observed among groups A, C, D and E rats.

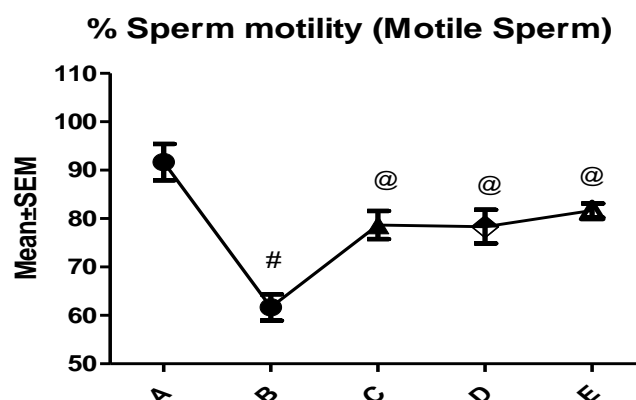


Figure 3: Showed comparison in motile sperm (%) among the groups after the administration of Valorphin, zinc, and ascorbate. # = Comparison with Group A; @ = Comparison with Group B. (n=5).

Superoxide Dismutase (SOD) mean values indicated in Figure 4 showed high significant decreased in SOD concentration in group B rats ($***p<0.001$) when compared with groups A, C, D and E. However, no significant difference in SOD concentration among groups A, C, D and E rats.

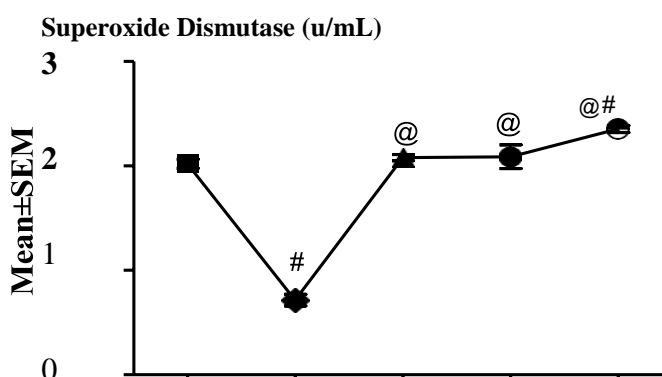


Figure 4: Showed comparison in Superoxide Dismutase Concentration (u/mL) among the groups after the administration of Valorphin, zinc, and ascorbate. # = Comparison with Group A; @ = Comparison with Group B. (n=5).

Result from Figure 5 revealed serum Glutathione Peroxidase (Gpx) concentration mean values after the administration of

Valorphin, zinc, and ascorbate. High significant decreased in Gpx concentration was observed in Valorphin only treated group B (** $p < 0.001$) when compared with groups A and E. More also, significant increase in serum Gpx concentration was also observed in Groups E (* $p < 0.05$) when compared with control group A. There was no significant difference in Gpx concentration among groups A, C and D.

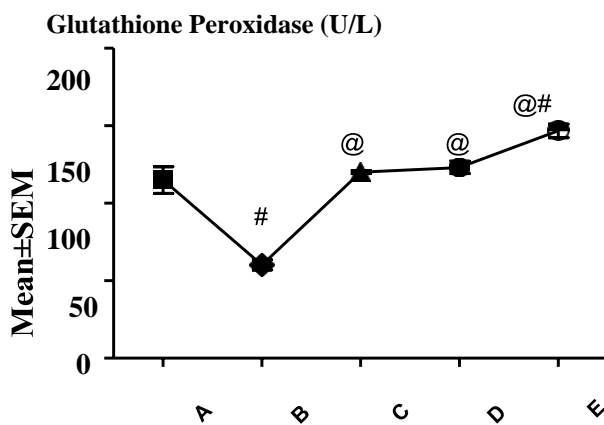


Figure 5: Showed comparison in Glutathione Peroxidase Concentration (U/L) among the groups after the administration of Valorphin, zinc and ascorbate. # = Comparison with Group A; @ = Comparison with Group B. (n=5).

Histological Analysis

Figure 6 shows the histological representation of all the groups administered with drugs including the control. It can be observed that the lumen of the control Group and group E are full of spermatozoa cells while the integrity of their germinal cells is intact. Group B shows arrest of germinal cells at the spermatocyte stage of spermatogenesis while the lumens of the group B rats were devoid of spermatozoa. Group C and Group D both show fair degenerative change with a fair number of spermatozoa in their lumen.

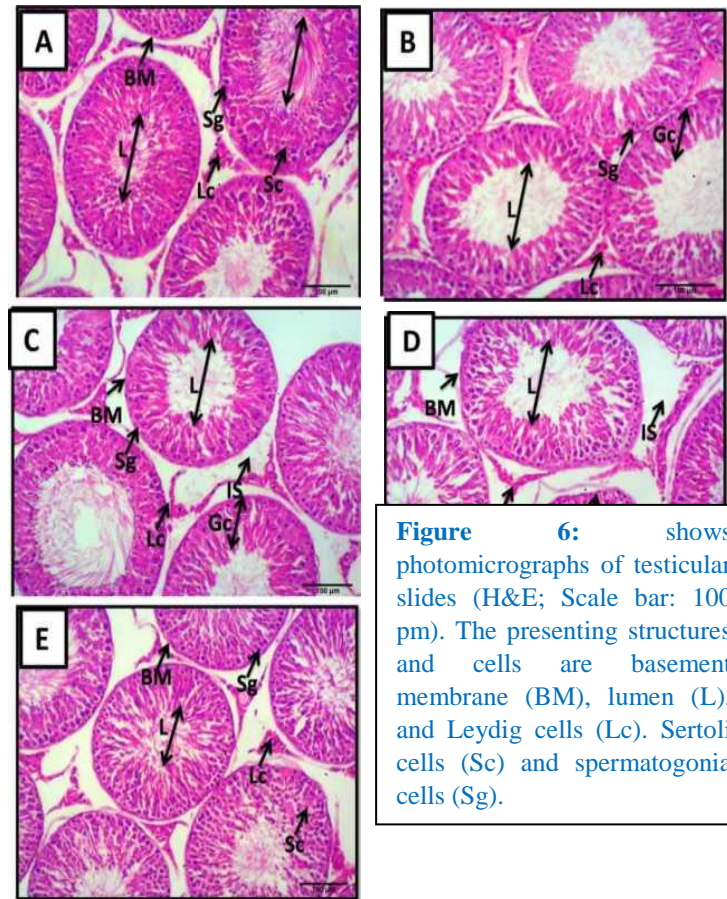


Figure 6: shows photomicrographs of testicular slides (H&E; Scale bar: 100 µm). The presenting structures and cells are basement membrane (BM), lumen (L), and Leydig cells (Lc). Sertoli cells (Sc) and spermatogonia cells (Sg).

DISCUSSION

Male factor infertility is responsible for about 40–50% of all infertility cases.¹ In cases where chronic opioid use in the male is implicated as the cause of infertility, the couple as with other marriages may be prone to worries and frequent internal wrangling because of infertility leading to divorce and separation.

Studies show that opioids affect the endocrine system, specifically the hypothalamus, and pituitary. This leads to a drop in gonadotropins like follicle-stimulating hormone and luteinizing hormone.⁸ Opioids also diminish testosterone production, a key hormone in male and female reproduction.^{9,10} It is pertinent to state that opioids cannot be entirely done away with, as they are vital painkillers and are essential in reducing and alleviating pain in critical disease states or even after surgery. Valorphin is among these opioids. It is a naturally occurring, endogenous opioid and it can also be synthesized. It works through the *mu* receptor just like most opioids and morphine. Like other opioids that work on the hypothalamus

and pituitary gland, in order to reduce the production of vital reproductive hormones such as FSH, LH, and testosterone, its adverse effects have been reported on the hypothalamic-pituitary-testicular axis, sperm function, and testicular structure.¹¹

Our findings are in line with all the above studies that implicate the effect of Valorphin on the testes, sperm parameters and testosterone. Group B showed a reduced concentration in testosterone and in the percentage of motile sperms although its effects were ameliorated by the administered antioxidants (zinc and vitamin C). Zinc is a key mineral that cells use to metabolize nutrients. Immune function, DNA and protein production, and cell division are all related to zinc levels in the body. Zinc also enables the male body to produce testosterone. Because of this, the levels of zinc may affect erectile function.¹²

Vitamin C has been shown to protect the body's cells from damage by oxidative stress and free radicals through its antioxidant functions. Vitamin C is one of the most abundant antioxidants in the semen of fertile men, and it contributes to the maintenance of healthy sperm by protecting the sperm's DNA from free radical damage.¹³ There is also some evidence that vitamin C supplements may improve semen quality. A study in infertile men showed that taking 1,000 mg vitamin C supplements twice a day for up to 2 months increased sperm motility by 92% and sperm count by more than 100%.¹⁴ Our results on sperm motility and testosterone concentration showed that zinc and vitamin C effectively served as protective and therapeutic aids against the administered Valorphin in the groups where vitamin C and zinc were co-administered with Valorphin.

The androgen receptor (AR), a member of the nuclear receptor superfamily, controls the expression of target genes by acting as a ligand-inducible transcription factor.¹⁷ When testosterone or its metabolite 5-dihydrotestosterone (DHT) binds to the AR, the receptor dimerizes, making it easier for the AR to connect to co-regulators and stimulate

the expression of target genes.¹⁹ A variety of reproductive damage results from the mutant receptor's inability to activate its target genes.²⁰ A study found that morphine effectively increased 5-alpha reductase mRNA expression in the liver and aromatase mRNA expression in the brain and gonads affect testosterone concentration in the body system.¹⁸ Findings from this study showed a significant down-regulation in Androgen Receptor Gene in Group B while a significant up-regulation of the Gene was observed in Group E when both groups were compared with control Group A. There was also a significant up-regulation in Androgen Receptor Gene expression in Groups C, D, and E when compared to Group B.

Superoxide dismutase (SOD) serves as the first line of antioxidant defense against ROS.²⁴ During oxidative stress, excess free radicals can damage biological cells. SOD is an enzyme that alternately catalyzes the dismutation of the superoxide radical into ordinary molecular oxygen, and hydrogen peroxide. Glutathione Peroxidase (Gpx) are a group of enzymes that protect the body from oxidative damage.²³ The activities of SOD and Gpx in this study were significantly lower in the valorphin treated group B when compared with the control group A and group E. Significant increase in SOD and Gpx concentration were observed in groups C, D, and E when compared to Group B. Decreased in SOD and Gpx concentration as observed in this present study is in alignment with Błaszczyk et al., who reported diminutions of Gpx activities in rabbits treated with naloxone and morphine.²² This shows that after the use of Naloxone to tone down the effect of the morphine there was still a slight decrease in Gpx activity. Furthermore, Paolo et al., reported significant decrease in antioxidant activities in opioid dependent patients while Toktam reported the protective effects of zinc and vitamin C as an antioxidant against Chemotherapy-Induced Male Reproductive Toxicity.^{25,26}

Ghoneim et al., reported that chronic usage of tramadol induced arrest of spermatogenesis together with sloughing of the germinal epithelium, thickening of the basement membrane of the seminiferous tubules, and apoptotic changes.¹⁵ Furthermore, Abou El Fatoh et al. reported the presence of severe diffuse testicular degeneration without spermatozoa following long-term administration of tramadol.¹⁶ The spermatocytes were mostly necrotic. The histological results from this study which showed that degenerative changes were observed in the group administered with Valorphin only and their lumens were found lacking spermatozoa therefore evidencing apoptosis of the cell in response to the degenerative change in the germ cell caused by Valorphin are in agreement with the findings of the above studies. Opioids are involved in the response of the immune system to stress and can induce immunosuppression, and one of the possible mechanisms of its implementation is apoptosis.²⁷

CONCLUSION

Valorphin has been shown to have a negative effect on the testes majorly by its influence on the hypothalamo-hypophyseal-gonadal axis. It influences the mRNA gene expression of the Androgen Receptor which in turn alters the effect of androgen on the body. This undesirable effect of valorphin was effectively ameliorated by the administration of Zinc and vitamin C.

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