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ORIGINAL ARTICLE

The Effect of Quinine and Ascorbic Acid on Rat Testes

L'effet de quinine et l'acide ascorbique sur un.un de testicules de rat.

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ABSTRACT

BACKGROUND: **We have previously demonstrated that quinine is a testicular toxicant in Sprague-Dawley rat.**

OBJECTIVE: **To describe the changes in the testicular levels of testosterone and lipid peroxidation secondary to quinine and ascorbic acid administration in rats.**

METHODS: **Twenty male Sprague-Dawley rats per group were assigned to one of three treatment groups: 0 mg quinine and 0 mg ascorbic acid /kg body weight (control); 10 mg quinine / kg BW; and 10 mg quinine plus 0.1 mg ascorbic acid /kg BW. Rats were intramuscularly administered their respective doses of quinine five days in a week and ascorbic acid three days in a week for eight weeks. All the animals were sacrificed at the end by decapitation. Seminal analysis was performed on tubular fluid from caudal epididymides. Evaluations were made for testicular levels of testosterone and lipid peroxidation through malondialdehyde (MDA). Testicular specimens were also processed for histology under light microscopy.**

RESULTS: **Quinine significantly ($p < 0.01$) increased free radicals (from elevation of MDA) and decreased testosterone in the testis compared with those of the control group and those treated with a combination of quinine and ascorbic acid. The semen of rats treated with only quinine demonstrated a significantly ($p < 0.001$) lower sperm concentration and motility compared to the controls and those treated with quinine plus ascorbic acid . Microscopic examination of cross-sections of seminiferous tubules also showed that ascorbic acid partially protected against quinine -induced testicular effects.**

CONCLUSION: **Ascorbic acid has beneficial effect and protects against quinine-induced testicular reduction of testosterone.**

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Keywords: **Quinine; ascorbic acid; testosterone; malondialdehyde; testis; rat**

RESUMÉ

Contexte: *Nous avons démontré précédemment cette quinine est un produit nocif testiculaire dans le rat de Sprague-Dawley.*

Objectif: *Pour décrire les changements dans les niveaux testiculaires de peroxidation de testostérone et lipide secondaire à la quinine et l'administration acide ascorbique dans les rats.*

Methodes: *Vingt rats mâles de Sprague-Dawley par le groupe ont été assignés à un de trois groupes de traitement : 0 quinine de mg et 0 mg l'acide ascorbique/le poids de corps de kg (le contrôle) ; 10 quinine de mg/BW de kg ; et 10 quinine de mg plus 0,1 mg l'acide ascorbique/ BW de kg. Les rats ont été intramusculairement administrés leurs doses respectives de quinine cinq jours dans une semaine et un acide ascorbique trois jours dans une semaine pour huit semaines. Tous les animaux ont été sacrifiés à la fin par la décapitation. L'analyse déterminante a été exécutée sur le liquide tubulaire d'epididymides caudal. Les évaluations ont été faites pour les niveaux testiculaires de peroxidation de testostérone et lipide par malondialdehyde (MDA). Les spécimens testiculaires ont été aussi traités pour l'histologie sous la microscopie légère.*

Resultats: *La quinine significativement ($p < 0,01$) a augmenté des radicaux libres (de l'élévation de MDA) et la testostérone diminuée dans le testicule comparé à ceux-là du groupe de contrôle et ces traité avec une combinaison de quinine et l'acide ascorbique. Le sperme de rats a traité avec seulement la quinine a démontré un significativement ($p < 0,001$) abaisse la concentration de sperme et motility a comparé aux contrôles et ces traité avec la quinine plus l'acide ascorbique. L'examen microscopique de coupes transversales de tubules seminifères a montré aussi cet acide ascorbique partiellement protégé contre la quinine -a persuadé des effets testiculaires.*

Conclsuion: *L'acide ascorbique a l'effet avantageux et protège contre la quinine -la réduction testiculaire persuadée de testostérone.*

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Mots cles : La quinine ; l'acide ascorbique ; la testostérone ; malondialdehyde ; le testicule ; le rat.

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Abbreviations: AA, Ascorbic acid; QAA, quinine and ascorbic acid; MDA, Malondialdehyde; TT, testosterone.

INTRODUCTION

Quinine is an alkaloid derived from Cinchona bark. Up till the development of chloroquine, quinine was the first line of drug in the treatment of malaria.[1] quinine is a potent blood schizonticidal drug against the four plasmodia species and is presently the treatment of choice for cerebral and complicated *Plasmodium falciparum* malaria[2] and nocturnal leg cramps.[3]

We have previously demonstrated that quinine is a testicular toxicant in Sprague-Dawley rat and had indicated that the disruption of synthesis of testosterone might be a mode of the deleterious effect of this anti-malaria drug[1] on the testis. However, the role of ascorbic acid , a free radical scavenger, on testicular concentration of testosterone in the presence of quinine is yet to be investigated. The testis is sensitive to a variety of stressors, such as hyperthermia, radiation and exposure to agents that induce apoptosis of germ cells[4]. Because oxidative stress in the testis is one of the major factors that induces germ cell apoptosis, this organ has fairly high concentrations of antioxidants, such as ascorbic acid and vitamin E[4]. These antioxidants protect germ cells against oxidative DNA damage[4], and play important roles in spermatogenesis. In fact, deficiency of ascorbic acid and vitamin E have been reported to cause the disturbance of spermatogenesis.[5,4].

Some testicular toxicants, such as cyclophosphomide, methoxychlor and aflatoxin B have been shown to partly exert their toxic effects by inducing a perturbation of the oxidant status of the testis towards pro-oxidants.[6] Studies have also demonstrated direct correlation between total antioxidant capacity of seminal plasma and sperm motility and these have suggested a potential protective role of anti-oxidant toward sperm motility.[7] Orchidectomy in rats has been shown to cause a significant decrease in blood concentration of ascorbic acid (a potent antioxidant).[8]

Oxidative stress is induced by reactive oxygen species, such as free radical (superoxide O^{2-}, hydroxyl OH, nitric oxide NO) and non radical (hydrogen peroxide H_2O_2) derivatives of oxygen. Conversely several physiological defence systems, enzymatic (catalase, superoxide dismutase, glutathione peroxidase) or non enzymatic such as thiols (glutathione, taurine, methionine), vitamins (A, C, E) or minerals (magnesium, selenium) are involved in the prevention of reactive oxygen species formation, or in the interception of reactive oxygen species attack[9]. Free radicals contain unpaired electrons on the last shell and that makes them highly reactive and responsible for oxidative damage of lipids. Peroxidation of lipid forming cell membranes causes changes in their structure and, in turn, the dysfunction of affected cells. Lipid peroxidation being a most spectacular manifestation of cell damage causes the transformation of membrane polyunsaturated fatty acids to hydroperoxides and degradation to low molecular species. One of the most important of these products is malondialdehyde (MDA) which is an indicator of free radical activity.[10]

There is no published work that has reported the effect of oxidant-antioxidant imbalance in the environment of seminiferous tubules of either man or animal on testicular TT concentration following quinine administration. The most widely used method to access extent of lipid peroxidation is the thiobarbituric acid reaction with MDA, a toxic byproduct of lipid peroxidation.[11] This method is of particular interest because of its procedural simplicity and nanomolar sensitivity.

In the present study, we investigated the effects of quinine and ascorbic acid on testicular levels of MDA and TT.

MATERIALS AND METHODS

Sixty adult male Sprague-Dawley rats weighing 180-200g were used for the experiments. They were procured from the Animal House of the College of Medicine, University of Lagos. They were allowed to acclimatize and maintained under standard photoperiodic condition in the Rat Room of the Department of Anatomy for two weeks. They were allowed unrestricted access to rat chow and pipe-born water in the Anatomy Department. The rats were weighed, and randomly divided into three groups of 20 rats each. Group 1 rats had quinine (10 mg/kg body weight per day; 5 days in week), group 2 rats had quinine (10 mg/kg body weight per day; 5 days in a week) and ascorbic acid , (0.1 mg/kg body weight per day; three days in a week), while group 3 constituted the controls and were administered equal volume of distilled water. All modalities of treatment were intramuscularly administered for duration of eight weeks. All animals were sacrificed by decapitation at the end of 8 weeks. Testes were excised and specimens were taken and processed for histology under light microscopy.

Estimation of sperm count and motility

The caudal epididymides of treated and control rats were incised and the fluid collected by a pipette. 5 μl of the epididymal fluid was delivered onto a glass slide and covered with a 22x22 mm cover slip. The slide was then examined under light microscope at a magnification of x400. Motility estimation was carried out at room temperature between 24 and 28°C (air-conditioned). The microscopic field was scanned systematically and each spermatozoon encountered was assessed and for the purpose of this study, motility was classified as either motile or non-motile. The procedure was repeated once and the average taken.

The sperm count was determined using the Neubauer improved haemocytometer. A dilution ratio of 1:20 from each well-mixed sample was prepared by diluting 50 μl of liquefied semen with 950 μl diluent. The latter was prepared by adding 50 g of sodium carbonate and 10 ml of 35% (v/v) formalin to distilled water and making up the solution to a final volume of 1000 ml.[12] Both chambers of the haematocytometer were scored and the average count calculated, provided that the difference between the two counts did not exceed 1/20 of their sum (i.e., less than 10% difference). When the two counts were not within 10%, they were discarded, the sample dilution re-mixed and another haemocytometer prepared and counted. To minimize error the count was conducted three times on each of the sample obtained from each epididymis.

The average of all the six counts (3 from each side) from a single rat was taken and this constituted one observation.

Estimation of testicular MDA level

MDA levels were determined in the supernatants of the testicular homogenates by the modified method of Buege and Aust[13]. Briefly, 0.25g testicular tissue sample was homogenized in 2.5 ml of 0.15M potassium chloride. The homogenate was centrifuged at 1000 g and the supernatant collected. An aliquot of 2 ml of 0.375% thiobarbituric acid, 25% 1 mol/L hydrochloride acid, and 15% trichloroacetic acid was added to 1.0ml of the tissue homogenate supernatant, mixed vigorously and heated for 15 minutes in a boiling water bath (80 – 90 °C). Subsequently, the samples were cooled down in ice-cold water and centrifuged at 1500 g for 15 minutes. Absorbance of the solution was taken at 535 nm against the reagent blank. Concentration was calculated using the molar absorptivity of malondialdehyde which is $1.56 \times 10^5 M^{-1} cm^{-1}$.

Estimation of testicular testosterone level

We homogenized 0.25g of testicular tissue sample in 2.5 ml of 0.15M potassium chloride. The homogenate was centrifuged at 1000 g and the supernatant collected testosterone concentration in supernatant was determined by the enzyme immunoassay technique based on the principle of competitive binding between testosterone in the test sample and TT-horseradish peroxidase conjugate for a constant amount of rabbit anti-testosterone , as reported previously[14]. All the animals were observed for general signs of drug toxicity (such as vomiting, diarrhoea, tremors, weakness, lethargy, refusal of feeds, weight loss, hair-loss, coma and death) throughout the duration of treatment.

All procedures involving animals in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals [15] and were approved by the Departmental Committee on the Use and Care of Animals.

Staistical Analysis

Data obtained from concentrations of MDA and TT, body weight, testicular weight, sperm motility and count from quinine -only, quinine plus ascorbic acid -treated rats, as well as those obtained from the distilled water-treated control animals were pooled and expressed as mean ± SD (standard deviation). The difference between distilled water-treated and test means was analyzed statistically by Student's *t*-test. Values of $p < 0.05$ were taken to imply statistical significance.

RESULTS

General and weight changes

There were no apparent signs of general toxicity observed in any of the animals throughout the duration of our observation.

Body and testicular weight. There were no statistically significant differences in the mean weights of rats in all the three groups at the beginning and end of the experiments . There was, however, statistically significant ($p<0.01$) differences in the mean testicular weights of the rats that had quinine only and those of the control and quinine plus ascorbic acid -treated groups (Table 1).

Testicular effects and Sperm motility and count

Quinine significantly ($p < 0.001$) reduced both sperm motility and count, while ascorbic acid significantly ($p < 0.001$) protected against the deleterious effects of quinine on these two parameters (Table 2).

Testicular MDA and testosterone **levels.**

There was a significant ($p < 0.01$) elevation of MDA levels in the supernatants of testicular homogenates of rats treated with quinine only compared with those of the control group and those treated with a combination of quinine and ascorbic acid . Rats treated with only quinine had significantly ($p < 0.01$) lower levels of testicular testosterone , while co-administration of ascorbic acid with quinine offered significant ($p < 0.01$) protection against testosterone reduction (Table 2).

Histology

The cross-sections of the seminiferous tubules of the control rats were fairly oval and regular in outline with normal seminiferous epithelium and numerous spermatozoa within their lumen (Figure 1). The seminiferous epithelium and interstitium of the seminiferous tubules of rats treated with only quinine were grossly distorted. Spermatozoa

Table 1: Effects of Quinine And Ascorbic Acid on Body and Testicular Weight of Rats

Group	Mean±SD Weight in grams			
	Initial BW	Final BW	% change BW	Testes
Distilled water	187.8 ± 9.8	240.6 ±15.2	52.99 ± 9.28	1.65 ± 0.16
Quinine	188.8 ± 9.2	220.5 ±12.1	31.94 ± 5.44	0.74 ± 0.09*
QAA	190.2 ± 9.9	230.4 ±14.5	40.67 ± 7.59	1.55 ± 0.15

BW, Body Weight

Table 2: Effects of Quinine and Ascorbic Acid on Testicular Testosterone, Malondialdehyde, Sperm Count and Motility in Rats

Group	Mean ± SD Testicular Effect			
	Testosterone (x10 ng/g of Testis)	MDA (μmol/g of Testis)	Sperm Count ($x10^6$/ml)	Sperm Motility (%)
Water	3.45 ± 0.89	0.40 ± 0.05	165.45 ±10.34	97.62 ± 1.94
Quinine	1.02 ± 0.33	1.07 ± 0.30	29.09 ± 3.96†	6.96 ± 2.03†
QAA*	2.95 ± 0.55	0.48 ± 0.05	141.29 ± 8.07	93.61 ± 4.52

Values given represent the mean ± SD of 20 observations, †$p<0.01$. ; *QAA, quinine and ascorbic acid; MDA, Malondialdehyde .

were scanty in the seminiferous tubular lumen (Figure 2). The seminiferous tubules of the rats that had both quinine and ascorbic acid showed only minimal distortion in their cytoarchitecture and numerous spermatozoa were present in their lumen (Figure 3).

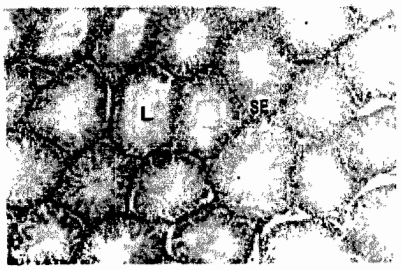

Figure 1: Cross-section of the seminiferous tubules of control rats. Stains: haematoxylin & eosin. Magnification: x 100. SE, seminiferous epithelium; L, lumen of seminiferous tubule, I = testicular interstitium.

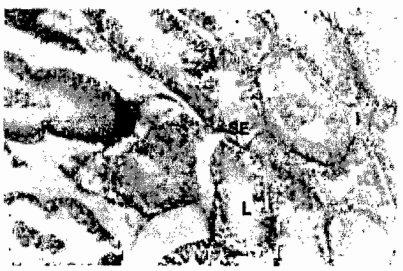

Figure 2: Cross-section of the seminiferous tubules of of rats treated with quinine for eight weeks. Stains: haematoxylin & eosin. Magnification: x 100. SE = seminiferous epithelium, L = lumen of seminiferous tubule, I = testicular interstitium

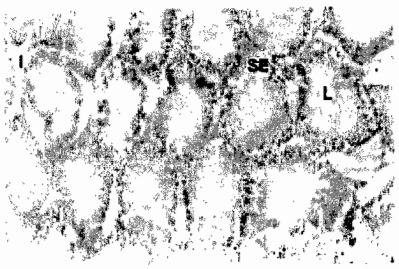

Figure 3: Cross-section of the seminiferous tubules of rats treated with quinine and ascorbic acid for eight weeks. Stains: haematoxylin & eosin. Magnification: x 100. S = seminiferous epithelium, L = lumen of seminiferous tubule, I = testicular interstitium.

DISCUSSION

The results of this present study showed that quinine caused a significant reduction in testicular TT and elevation of testicular MDA concentrations that were largely prevented by ascorbic acid . We observed that there was a disruption of the seminiferous epithelium and testicular interstitium in testes of rats administered quinine only while concurrent treatment of ascorbic acid with quinine significantly prevented these quinine -induced testicular toxic effects.

Up to 60% of infertile couples have difficulty conceiving due to "male factor" subfertility[16], meaning one or more of the sperm parameters are abnormal. The production of abnormal quantities of reactive oxygen species is thought to be involved in many facets of human male infertility.[17] Sperm exposed to superoxide anions are apparently rendered dysfunctional by lipid peroxidation and altered membrane function, along with impaired metabolism, morphology, and motility.[18] The formation of reactive oxygen species has also been associated with decreased sperm-egg interaction and reduced fertility.[19] Ascorbic acid has been given to infertile men for years, as it has anecdotally proven to be efficacious in improving sperm parameters.[20] Studies have documented the efficacy of antioxidant treatment on human spermatozoa and fertilization rates, especially in the setting of *in-vitro* fertilization.[21]

Steroidogenesis in the testes is under the physiologic control of two dehydrogenases: 3β-and 17β-hydrosteroid dehydrogenases. Both dehydrogenases are directly involved in biosynthesis of testosterone rom pregnenolone as well as androstenedione. A constant supply of cholesterol and ascorbic acid is required for the synthesis of steroid hormones.[22] ascorbic acid , an easily diffusible water-soluble biologic reductant, is found in abundance in testes, where it plays an important role in testicular hormonogenesis.

In-vitro studies have revealed that ascorbic acid significantly stimulates testicular steroid dehydrogenases and TT content in rat testis.[23] Ascorbic acid increases the conversion of pregnenolone to delta 4 steroids and decreased its conversion to delta 5 steroids in guinea pig (delta 4 steroids yield more TT than delta 5 steroids).[24] In present study, the protection of ascorbic acid against quinine -induced depletion of testicular TT and toxicity shows that quinine partly induced its deleterious effect on the testis by causing a disruption in the function of ascorbic acid , a cofactor in many biological reactions, including testicular hormonogenesis.

Elevation of MDA levels is a reflection of increased oxidative stress and provides evidence of lipid peroxidation[25]. Free radicals react with cellular macromolecules and have a destructive effect on lipids of all membranes. Consequently cellular destruction, particularly in membranes, is seen. The end product of this phenomenon called lipid peroxidation is MDA and the latter is a well known and generally acceptable reliable indicator of lipid peroxidation[26]. The free radicals react with lipids in cell and mitochondrial membranes, forming lipid peroxides. Peroxidation of membrane lipids changes membrane permeability and disrupts membrane integrity, impairing cell integrity[27]. Significant changes in structure and function of membranes result in cell death via apoptosis[28]. The spermatogenic epithelial damage induced by quinine could therefore have arisen through generation of free radicals that induced membrane lipid peroxidation or by MDA itself forming bonds with nucleic proteins as MDA has been reported to be capable of reacting with DNA to form adducts and cross-links[29].

The low sperm count observed in the epididymal fluid of rats treated with quinine only is unarguably secondary to the destruction of the seminiferous epithelium. However, a direct effect of the free radicals generated by quinine on the spermatozoal membrane might have contributed to the poor motility of the spermatozoa. The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from those of mammalian somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated

fatty acids therefore sperm cells are particularly susceptible to the damage induced by free radicals[30] and by extension are highly probable targets for quinine . Thus, this at least in part, explains the significantly ($p < 0.001$) low sperm motility observed in rats treated with quinine only when compared to the control rats and those treated with quinine and ascorbic acid , a potent free radical scavenger.

In this study, we show that applying ascorbic acid with quinine significantly diminishes testicular testosterone reduction, histological damage in the seminiferous tubules and low sperm count and motility.

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