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Quinine lowers serum and testicular testosterone in adult Sprague-Dawley rats

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Summary
We have earlier demonstrated that quinine (QU) is a testicular toxicant. This present study was aimed at evaluating the effects of QU on both the serum and testicular levels of testosterone (TT) in an attempt to elucidate one of the potential mechanisms of QU-induced testicular toxicity. Thirty adult male Sprague-Dawley rats weighing 180-200g were used and were randomly divided into 3 groups of 10 rats each. Rats in group 1 had distilled water. Rats in group 2 had QU only at the dose of 10 mg/kg body weight per day (5 days in a week) for 8 weeks. Rats in group 3 rats had 10 mg/kg of QU (5 days in week) and 0.05 mg/kg of TT (3 days in a week) for 8 weeks. All the animals were sacrificed at the end of 8 weeks by decapitation. Seminal analysis was done on the tubular fluid aspirated from the caudal epididymides. The two testes were excised, weighed, and volume estimated. One testis of each rat (0.25 g of tissue) was homogenized with Potassium Chloride and TT level determined in the supernatant of the homogenate, while the other testis was processed for histology. Morphometry was carried out by assessing the diameter, cross-sectional area, number of profiles per unit area, length density and numerical density of the seminiferous tubules, and the relative and absolute volume of testicular components. The serum levels of TT in all the animals were also determined at the time of sacrifice. Both the serum and testicular levels of TT in rats administered QU only were significantly ($P < 0.001$) lower than those of the control and QU plus TT-treated rats. We conclude that QU induces spermatogenic epithelial toxicity by possibly interfering with the steroidogenic function of the Leydig cell.

Keywords: *Quinine, testosterone, seminiferous tubules, Leydig cells, testicular interstitium*

Résumé
Cette étude avait pour but d'évaluer les effets de la quinine sur les taux de testostérone testiculaire pour élucider un des potentiels mécanismes de la toxicité testiculaire induite par la quinine. Trente souris male de Spargue-Drawley pésant entre 180-200g étaient utilisés et divisés au hasard dans 3 groupes de 10 souris chacun. Les souris dans le groupe 1 recevaient de l'eau distillée uniquement ; ceux du groupe 2 recevaient de la quinine à la dose de 10mg /kg de poids corporel pendant 5 jours dans la semaine et 0.05mg/kg de testostérone testiculaire pendant 3 jours de la semaine pour 8 semaines. Tous les animaux étaient sacrifiés à la fin de la 8ieme semaine par décapitation. L'analyse séminale était faite sur le fluide tubulaire aspiré des épididymes caudaux. Les 2 testicules étaient excisés, pésés et le volume estimés. Un testicule de souris (0.25g de tissue) était homogénéisé avec le chlorite de potassium et le taux de testostérone testiculaire déterminé dans le supernatant homogénéisé alors que les testicules étaient analysés histologiquement. La morphométrie était faite pour évaluer le diamètre, surface, nombre de profile par unité de surface, densité longue et densité numérique des tubules séminifères, et le volume testiculaire des constituents. Les taux du sérum et de la testostérone testiculaire chez ces animaux étaient déterminés à l'heure de leur sacrifice. Les résultats des taux du sérum et du taux de testostérone testiculaire chez les souris administrés à la quinine étaient seulement significativement plus baisse que les taux observés au contrôle et au groupe traité de la quinine plus testostérone testiculaire. Nous avons conclu que la toxicité épithéliale spermatogénique induite par la quinine interfère probablement avec la fonction steroidogénique des cellules de Leydig.

Introduction

The antimalarial quinine (QU) still remains an important drug of choice for the treatment of severe and complicated malaria [1]. It is now widely used in the treatment of nocturnal cramps [2, 3]. QU is a quinoline-methanol derived from the bark of the *Cinchona* tree. It has been used in the suppression and treatment of malaria for more than three centuries [4] and is one of the oldest drugs in the pharmacopoeia [5].

Although we have previously reported that QU is a testicular toxicant, causing a general destruction of both the seminiferous epithelium and the testicular interstitium in rat [6], much less is known about the mechanisms leading up to this disruption. The cells responsible for protection of germ cells, the Sertoli cells and the principal cells of the epididymis are androgen-dependent [7]. A decrease in androgen level may result in the breakdown of the blood-testis barrier and blood-epididymis barrier [8]. Leydig cells are the primary source of testicular androgens in the mammalian male and TT is necessary to maintain spermatogenesis [9].

Results of research work in our laboratory [10, 11] and others [12, 13] suggest that Leydig cell may be the target cell of testicular toxicants while another report demonstrated that the administration of TT at a dose of 0.05 mg/kg body weight, three times in a week for 10 weeks to male rats exposed to chloroquine (a 4-aminoquinoline) is

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protective against the antifertility action of chloroquine [14] suggesting that the administration of chloroquine and the consequent reduction in TT production may therefore be held responsible for this chloroquine-induced changes in the testis. Identifying chemicals that alter endocrine function is a high public health and research priority; due to the potential risk such chemicals pose to the reproductive health of humans [15].

In this study, we present the results of TT assays in serum and testes and seminal fluid analyses, as well as morphometric data obtained by the use of test probes on isotropic uniform random (vertical) sections of the testes of Sprague-Dawley rats exposed to QU and TT.

Materials and methods

The protocols of this animal study were approved by the Departmental Animal Research Committee. Thirty 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 10 rats each. Rats in group 1 had distilled water for 8 weeks. Rats in group 2 had 10 mg/kg body weight per day of QU (5 days in a week) [16], while those in group 3 had in addition to QU, 0.05 mg/kg body weight per day of TT, (three times in a week) [14]. Each treatment modality was administered intramuscularly for 8 weeks and all animals were sacrificed on the last day of injection by decapitation [17]. QU was administered for 8 weeks for two reasons. Damage to spermatogonia by toxic agents will take at least 56 days for the number of spermatozoa in the seminiferous tubular lumen and epididymal fluid to be reduced in rat since the duration of spermatogenesis in this mammal is between 51.6 and 56 days [17]. Secondly, QU is presently the treatment of choice for nocturnal leg cramps and its use in this condition frequently takes longer than 8 weeks [3, 18, 19]. The caudal epididymides of all animals were incised, sperms allowed to swim out and motility assessed while the spermatozoa were suspended in normal saline [20]. The sperm count was determined using the Neubauer improved haemocytometer. To minimize error the count was repeated at least five times for each rat and the mean taken. The testes were carefully dissected out, trimmed of all fat and blotted dry to remove any blood. Their weight was noted and volume measured by water displacement and then fixed in 10% formol-saline. The tissues were dehydrated in graded series of ethanol after which they were cleared in xylene. Once cleared, the tissues were infiltrated in molten paraffin wax in the oven at 58°C. Prior to embedding, it was ensured that the sections to be cut by the microtome were orientated perpendicular to the long axes of the testes. These sections were designated "vertical sections". Serial sections of 5μm thick were obtained from a solid block of tissue and were stained with haematoxylin and eosin stains.

Stereological analysis

For each testis, five vertical sections from the polar and the equatorial regions were sampled and an unbiased numerical estimation of the following morphometric parameters determined using a systematic random scheme:

(a) *Diameter of seminiferous tubules (D)*
The mean tubule diameter (*D*) was derived in 25 round transverse sections of seminiferous tubules per animal by taking the average of two diameters, D_1 and D_2 at right angles where the ratio of $D_1 / D2 \geq 0.85$.

(b) *Cross-sectional area of the seminiferous tubules (Ac)*
Cross-sectional area (*Ac*) of the seminiferous tubules was determined using the equation $A_c = \partial D^2/4$, where ∂ is equivalent to 3.142 and *D* the mean diameter of the seminiferous tubules.

(c) *Number of profiles of seminiferous tubules in a unit area (N_A)*
The number of profiles of seminiferous tubules per unit area was determined using the unbiased counting frame proposed by Gundersen [21].

(d) *Length density (L_v) of seminiferous tubules*
The length density of the seminiferous tubules was obtained by applying the equation for a tube model as previously described [22]. Briefly the length of a structure is represented as profiles of the structure and is sampled uniformly by isotropic area probes. The formula below for estimating length density, L_v, relies on the simple fact that test planes 'feel' curve length: $Lv = 2 \times N_A$, where N_A is the number of profiles per two-dimensional test frame area.

(e) *Volume density (Vv) of testicular components*
Volume density was determined using the principle of point counting. Briefly the ratio of the number of the points hitting each testicular component was obtained by dividing the sum of the points falling on each component by the total number of points counted [23]. This ratio was multiplied by 100 to give the percentage of volume density.

(f) *Absolute volume of each testicular component*
The absolute volume of each testicular component was obtained by multiplying its relative volume (volume density) by the testicular volume obtained by the water displacement method.

(g) *Numerical density (Nv) of seminiferous tubules*
This is the number of profiles per unit volume and it was

determined using the modified Floderus equation: $N_V = N_A / (D + T)$ [24] where, N_A is the number of profiles per unit area, D is the mean diameter of the seminiferous tubule and T the average thickness of the section.

Testicular homogenate

Testicular tissue sample (0.25g) was homogenized in 2.5 ml of 0.15M potassium chloride. The homogenate was centrifuged at 1000 g and the supernatant collected.

Testosterone assay

TT concentration in both the homogenate supernatant and blood were determined by the enzyme immunoassay technique based on the principle of competitive binding between TT in the test sample and TT- horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-TT, as reported previously [25].

Data analysis

Data obtained from QU-only treated subjects and QU plus TT-treated ones, as well as those obtained from the distilled water-treated control animals were pooled and expressed as mean±SD (standard deviation). The difference between the distilled water-treated test means and those of QU only and QU plus TT groups was analysed statistically by Student's *t*-test. Values of $p < 0.05$ were taken to imply statistical significance.

Results

Qualitative histological findings

Testicular sections from control rats displayed fairly consistently good histological preservation indicating that the fixation method and tissue processing was optimal. The cross-sections of the seminiferous tubules of the control rats were fairly circular or oval in outline with normal seminiferous epithelium and numerous spermatozoa within their lumen (Figure 1).

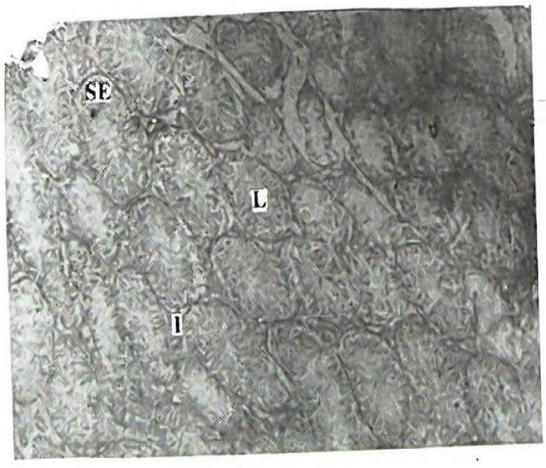

Fig. 1: *Cross-section of the seminiferous tubules of control rat*
S = seminiferous epithelium, L = lumen of seminiferous tubule, I = testicular intersititum
Stains: haematoxylin and eosin. Magnification: x 40

The seminiferous tubules of rats treated with QU only were grossly distorted. Their seminiferous epithelium was grossly diminished, and their interstitium was greatly distorted as well. Seminiferous tubular sections contained many sloughed cells. Though spermatids and spermatozoa were present within the seminiferous tubular lumen, they were very scanty when compared to the control and QU plus TT-treated animals (Figure 2). The seminiferous tubules of the rats administered QU plus TT showed only slight reduction in regularity of outline (compared to the controls), while the general destruction that characterized the cross-sections of seminiferous tubules of the rats treated with QU only was essentially absent (Figures 3).

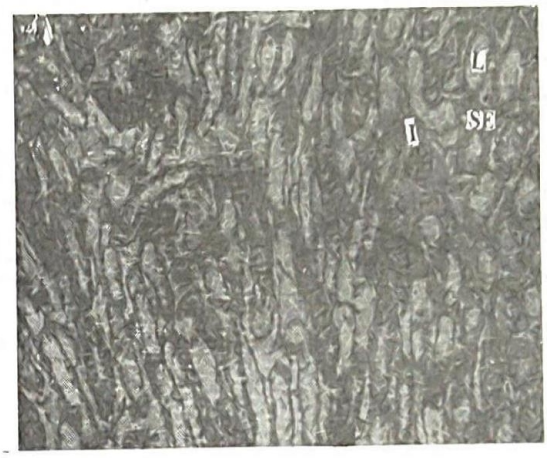

Fig. 2: *Cross-section of the seminiferous tubules of rats treated with quinine for 8 weeks.*
S = seminiferous epithelium, L = lumen of seminiferous tubule, I = testicular Interstitium
Stains: haematoxylin and eosin. Magnification: x 40

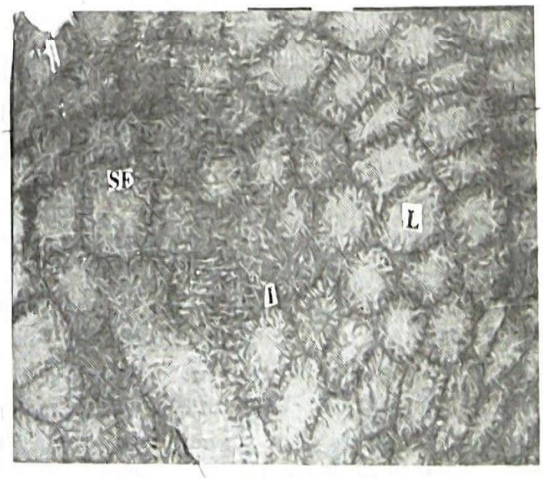

Fig. 3: *Cross-section of the seminiferous tubules of rats treated with quinine and testosterone for 8 weeks.*
S = seminiferous epithelium, L = lumen of seminiferous tubule I = testicular interstitium.
Stains: haematoxylin and eosin Magnification: x 40

Morphometric findings

The mean initial and final body weight of the control rats were 189.7 ± 9.7 and 230 ± 12.1; those of the QU-treated rats were 191.6 ± 8.5 and 222.4 ± 11.2, while those of QU plus TT- treated group were 189.5 ± 9.5 and 232.2 ± 12.9 g respectively. The differences between the final and initial body weight (i.e. body weight gain) of the control (41.2 g), QU-treated (30.8 g) and QU plus TT-treated rats (42.7 g) are not statistically significant.

Table 1: Effects of quinine on body weight of rats

Treatment group of rats	Initial body weight (g)	Final body weight (g)	Body weight difference (g)
1 = Distilled water	189.7 ± 9.7	230.9 ± 12.1	41.2
2 = Quinine	191.6 ± 8.5	222.4 ± 11.2	30.8
3 = Quinine and TT	189.5 + 9.5	232.2 ± 12.9	42.7

Values given represent the mean ± SD (standard deviation) of 10 observations

area of the seminiferous tubules of the QU-only treated groups were significantly ($p < 0.001$) reduced while their number of seminiferous tubules per unit area of testis, length and numerical densities were significantly ($p < 0.001$) elevated compared to those of the controls.

QU significantly reduced the relative ($p < 0.05$) and absolute volume ($p < 0.01$) of the seminiferous epithelium, while co-administration of TT with QU offered protection against the deleterious effects of the latter on seminiferous epithelium. There was a significant ($p < 0.05$) reduction in the absolute volume of the testicular interstitium of rats administered QU only compared to those of the control and QU plus TT-treated ones (Table 3).

Hormonal assays and seminal analyses

Both serum and testicular TT were significantly ($p < 0.001$) reduced by QU. Rats treated with QU only had significantly ($p < 0.001$) lower sperm motility and count when compared to those of the controls and QU plus TT-treated rats (Table 4).

Table 2: Effects of QU on TW, TV, D, A_C, N_A, L_V and N_V of seminiferous tubules

Group of rats	TW (g)	TV (ml)	D (μm)	A_C ($x10^3\ \mu m^2$)	N_A ($x10^{-8}\ \mu m^{-2}$)	L_V ($x10^{-8}\ \mu m^{-2}$)	N_V ($x10^{-10}\ \mu m^{-3}$)
1(DW)	1.59±0.15	1.57±0.14	219.09±17.21	37.90±5.51	33.96±2.97	68.11±5.56	15.35±1.86
2(QU)	0.79±0.11	*0.76±0.08*	88.78±8.89*	5.23±0.85*	86.24±8.98*	172.71±8.64*	93.07±7.85*
3(QU&TT)	1.60±0.18	1.58±0.17	220.05±18.11	38.24±5.95	33.01±2.95	66.31±5.02	14.99±1.91

*Values given represent the mean±SD (standard deviation) of 10 observations; *p < 0.001*
QU: Quinine
DW: Distilled water
TT: Testosterone
TW: Testicular weight
TV: Testicular volume
D: Diameter of seminiferous tubule
A_C: Cross-sectional area of seminiferous tubule
N_A: Number of profiles of seminiferous tubules per unit area of testis
L_V: Length density of seminiferous tubule
N_V: Numerical density of seminiferous tubule

There were statistically significant differences ($p < 0.01$) between the mean testicular weights and volumes; seminiferous tubular diameter and cross-sectional areas; number of profiles of seminiferous tubules per unit area of testis; length and numerical densities of seminiferous tubules of the QU only-treated and the control (as well as QU plus TT-treated) rats (Table 1). The testicular weight and volume; diameter and cross-sectional area of seminiferous tubules; number of profiles of seminiferous tubules per unit area of testis; length and numerical densities of the QU plus TT-treated rats were not significantly different from those of the controls (Table 2). The mean testicular weight, testicular volume, diameter and cross-sectional

Discussion

The present study demonstrated that administration of QU to Sprague-Dawley rats caused a reduction in both testicular and serum TT. In addition, the disruption to spermatogenesis (from both qualitative observation and morphometric parameters) was evident in testes of rats administered QU only while concurrent administration of TT prevents these QU-induced testicular deleterious effects. These findings are consistent with previous studies indicating TT could maintain spermatogenesis [26] or modulate QU-induced testicular damage [27].

Our results also showed that while QU also significantly lowered the sperm count and motility, concur-

Table 3: Efeects of quinine on testicular components of adult Sprague-Dawley rats

Treatment group of rats	SE		Intestitium		Lumen	
	V_v (%)	AV (ml)	V_v (%)	AV (ml)	V_v (%)	AV (ml)
1 = DW	75.00±4.62	1.19±0.20	12.50±0.92	0.20±0.03	12.50±0.91	0.20±0.03
2 = QU	65.51±3.98†	0.50±0.08*	20.24±3.45†	0.15±0.02†	14.25±1.01	0.11±0.01†
3 = QU & TT	74.50±4.99	1.19±0.19	13.50±1.02	0.22±0.06	12.00±0.91	0.19±0.05

*Values given represent the mean±SD (standard deviation) of 10 observations; †p<0.05; *p<0.01*
QU: Quinine
DW: Distilled water
TT: Testosterone
V_v (%): Percentage volume density
AV: Absolute volume
SE: Seminiferous epithelium

Table 4: Effects of QU on serum and testicular testosterone levels sperm motility and sperm count.

Treatment group of rats	Serum TT (ng/ml)	Testicular TT (x10 ng/g of Testis)	Sperm motility (%)	Sperm count ($x10^6$/ml)
1 = DW	0.36+0.02	3.47±0.91	99.0 ± 1.0	155.9 ± 6.9
2 = QU	0.13+0.05*	1.04±0.35*	5.0 ± 1.5*	25.0 ± 2.5*
3 = QU & TT	0.36+0.33	3.50±0.93	97.5 ± 2.4	154.9 ± 6.6

*Values given represent the mean±SD (standard deviation) of 10 observations (50 for sperm count): *p<0.001*
QU: Quinine
DW: Distilled water
TT: Testosterone

rent administration of TT appeared to have protected against QU toxic effects on sperm quality.

Though the mean volume density of the testicular interstitium of rats treated with QU was higher when compared to the control animals, the mean absolute volume of the interstitium was however significantly reduced ($p < 0.05$). The reason for the increase in the mean relative volume of the interstitium was as a result of the destruction of the seminiferous epithelium, the former therefore increasing at the expense of the latter. This notwithstanding the mean absolute volume of the interstitium still suffered an absolute reduction in size (0.15 ± 0.02 v 0.20 ± 0.03 ml). It is therefore substantially attractive to suggest that QU caused a reduction of testicular and serum TT secondary to the destruction of the testicular interstitium. As TT is indispensable to spermatogenesis, a depletion of the spermatogenic epithelium follows QU administration via a reduction of TT. It is interesting to note that when TT and QU were given together there was no evidence of testicular destruction, neither was there a low sperm count or motility. Consistent with our findings is the fact that testicular volume has been shown to be positively correlated with TT level [28] as well as testicular function [29].

Finally, the lack of significant differences between the body weight gain (differences in body weight of the rats at the end and beginning of the experiment) of the rats in the control and experimental groups; coupled with the presence of significant differences between the testicular weight of the QU-treated rats and the control group suggests that the reduced testicular weight in the QU-treated group is more likely to be QU-induced than a mere reflection of a general reduction in the body weight of the rats. This further suggests that the rat testis may be unusually susceptible or predisposed to QU-induced toxicity.

It should be emphasized that QU is still a widely utilized drug in humans [30]. This study suggests that further clinical studies are needed to determine the putative toxicological effects of QU on the testis and consideration of TT introduction in long term use of QU administration.

Conclusion

Based on our findings, it is clear that, QU-induced testicular toxicity is at least partly via a disruption in the steroidogenic function of the Leydig cell.

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