Chronic Chloroquine Administrations Cause Low Circulating Plasma Testosterone and Low Luteinizing Hormone Associated with Testicular Lesion in Rat

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Abstract

Chloroquine is an important drug in the malaria chemotherapy. It also has indication in treating rheumatoid arthritis, lupus erythematosus, extra intestinal amoebias and in reducing viral load in AIDS. Rats were daily given chloroquine (10mg kg^{-1}, ip), normal saline (10ml kg^{-1}, ip) as control. At days 7, 14 and 21, six rats on the weekly assessment were anesthetized and sacrificed. Cardiac puncture and circulating plasma testosterone and lutenizing hormone measured withdrew blood. Testes were excised and the histopathologies of the sectioned tissues were evaluated under microscopy.

In rats treated with chloroquine, there were testicular lesions and diminutive circulating plasma testosterone and plasma luteinizing hormone (LH). These effects could precipitate sterility as they affect spermatogenesis. It is suggested that chronic chloroquine administration could retard steroidogenesis in Leydig cells and spermatogenesis in Sertoli cells due to fibrosis of these tissues.

Key words: Chloroquine, testicular lesion, low circulating testosterone and luteinizing hormones.

Introduction

Chloroquine is a 4-aminoquinoline derivative, which presently is the mainstay of the antimalaria therapy in Africa despite occurrence of *Plasmodium falciparium* resistant strain. (Bloland *et al.,* 1990, WHO, 1996) Its other uses are in the treatment of extra-intestinal and intrahepahtic amoebiasis, rheumatoid arthritis and lupus erythematosus and recently reported to have broad spectrum anti-HIV activity (Savarino *et al.,* 2001). Chloroquine binds the ferriprotoporphyrine (ix) receptor during hemoglobin meal by malaria parasites (Chou *et al.,* 1980, Lacy and Peltit 1970) and bind to the parasite DNA by intercalation (Gamage *et al.,* 1994, Sartoruis and Schneider, 1997, Smith *et al.,* 1994, Washington *et al.,* 1973). Chloroquine binds to chromatin's, DNA histones and ribosome from mammalian tissues. The DNA intercalation is non-selective for malarial parasites as it occurs also with mammalian DNA. Thus protein synthesis and enzyme activities may be diminished or disrupted in sensitive tissues.

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Chloroquine may cause anti-fertility in female rats (Okanlowon and Ashiru, 1992) and its anti- fertility in male rats may result from reduction in the circulating plasma testosterone (Adeeko et al., 1992, Okanlawon et al., 1990). Testosterone is required for spermatogenesis and maturation of sperm. The Leydig cells through leutenising hormone receptor regulates testosterone synthesis. The Sertoli cells, somniferous tubules, interstitial cells also provide support for germinal cells to synthesize follicule stimulating hormones, androgen receptors and act as a blood-testis barrier (Goslar et al., 1982). It is therefore germane to study the testicular tissue status during chronic Chloroquine ingestion as it binds avidly to tissues.

Materials and Methods

Animals and experimental procedure: Male Wistar albino rats (4-5 weeks old weighing 180 – 200 g) were procured from the University of Uyo Animal House unit. The animals were housed in plastic cages and have free access to food and water. They were allowed 12h/12h light and dark cycle in a laboratory environment at 25^0C with 39% humidity where they acclimatized for 7 days before the study. In all experiment involving animals, local animal care committee approved the procedures. Animals were administered Chloroquine HCl (10mg/kg ,ip) daily for 21 days. The dose did not produce any mortality. The controls were administered with normal saline (10ml /kg, ip) daily for 21days at days 7, 14, and 21 of drug treatment, 6 rats were used for each group. Animals were anaesthetized with 0.3ml [(3.5% w/v Chloral hydrate)] and 5ml of blood were obtained by cardiac puncture. The animals were sacrificed and testes were dissected out and fixed in neutral 10% formalin for 2 days. From the 5ml of blood, about 3ml plasma was obtained after centrifugation at 3000g for 5 minutes. Samples of blood were stored in the freezer at -20^0C in the dark until analysis.

Testis tissue processing for light microscopy: Tissue blocks of testes fixed in neutral 10% formalin for 2 days were used for histological examination. All samples were embedded in paraffin. 5µm slices were obtained and stained with haemoxylin and eosin. Optical microscopy evaluation was carried out by a "blinded" pathologist.

Testis processing for cytochrome p450, cytochrome b5 and NADPH cytochrome c reductase assays: Testes tissues not used for histopathology examination were homogenized in ice-cold 0.22M tris buffer of pH 7.4 and 1mM EDTA. The microsomal testicular fractions were prepared by the method of Menard (et al., 1979). Protein contents were determined with a BCA protein Assay reagent (Pierce II). Cytochrome p450 and cytochrome b5 were measured according to Omura and Sato (1984). The NADPH cytochrome c reductase was measured by the method of Philips and Largdon (1962) using extinction coefficient of $19.6mn^{-1} cm^{-1}$.

Assay for testosterone: The plasma concentration of testosterone was determined by radioimmunoassay technique with reagents of Sorin (Belgium). Data were expanded in terms of nmole/L of rat. The testosterone used as reference preparation trace was purchased from Duport (Italy). All plasma samples were access in triplicates. The Intra-assay and inter-assay coefficient of variations were 4.5 and 6.7% respectively.

Assay for Leuteinizing hormone (LH): LH was determined by double antibody method of Midgley (1967) using Sigma reagents. Sensitivity of LH assay was 2ng/ml. The intra-assay coefficient of variation was 3.2%.

Drugs and chemicals: Chloroquine Hydrochloride (Lek, Slovenia) was commercially obtained. The batches used fulfilled the requirement of content uniformity tests according to standard quality control criteria of the USP 1996. Other chemicals and reagents were of highest purity (British drug House or May and Baker, England).

Statistical evaluation: Data were analyzed using ANOVA followed by Dunnetts t-test, p< 0.05 is indicative of significance.

Results and Discussion

Characteristic testicular lesion that is necrotic such as that of spermatogenia, spermatocytes and cap phase spermatids have been observed with several agents acting through different mechanisms (Russel *et al.,* 1998). Such necrotic features were also observed in our study with chronic chloroquine treatments (Table 1). The histopathology of the earlier testicular lesion found on day7 of chloroquine treatment was oedema of the interstitial cells, Leydig cells and capillary dilation. Day 14 showed necrosis of somniferous tubule cells oedema-based tubule and disappearance of Lumen with evidence of fibrosis (Table 1). At day 21, there were the necroses of the somniferous tubular cells, necrosis of the Sertoli cells and oedema of the basal lamina with the disappearance of lumen. Chloroquine therefore can acutely disturb the testosterone homeostasis via the alteration of the interstitial cells or Leydig cells at the initial onset and chronically with the necrosis of somniferous tabular cells and Sertoli cells. The low plasma testosterone level at days 7, 14 and 21 (Table 2) may therefore be as a result of the degeneration of Leydig cells which produce testosterone that is released into circulation unlike the testosterone produced from Sertoli cells which essentially is for spermatogenesis (Ishii-Ohba Maisumura *et al.,* 1984).

Table 1. Histopathology evaluation of the effect of chronic chloroquine administration on the testis.

TESTICULAR NECROSIS SCORE	
Duration of treatment	Histopathology evaluation
Day 0 (Normal saline)	6/6 (0)
Day 7 (Chloroquine)	3/6 (1)*
Day 14 (Chloroquine)	5/6 (3)*
Day 21 (Chloroquine)	6/6 (3)*

*P<0.05, compared to control (Normal)

The severity score of the lesion is shown in parenthesis.

Severity Score:
0   =   normal saline
1   =   Oedema of interstitial spaces Leydig cells and capillary dilatation.
2   =   Necrosis of semniferous tubule cells, disappearance of. Sertoli   cells, oedema of basal membrane
3   =   Loss of the original cellular structure of semniferous tubule and disappearance of the lumen.
Severity scores are averages based on the number of animals with lesions from groups of 6, each tissues slide preparation had three samples from each animal.

Sertoli cells produce insulin-like growth factors (IGF – binding proteins-3) (1GFBP-3). Chloroquine is an inhibitor of lysosomal enzyme activity in the internalization of proteins and receptors. Chloroquine inhibits IGFBP-3 reduction in Sertoli cells (Smith *et al.,* 1994)

143

thus influencing increase surface proteoglycan. This causes attendant disruption of the integrity of Sertoli cells with fibrosis. IGFBP-3 accumulation could also accentuate apoptosis of cells (Zahideh *et al.,*1992). Chloroquine may also inhibit lysosomal degradation of epidermal growth factor with the attendant accumulation of epidermal growth factor in cells which inhibits testicular steroidogenesis (Hush *et al.,* 1981) Chloroquine inhibits intracellular degradation of procollagen (Eletftheriades *et al.,* 1994) thus increasing the accumulation of collagen at the Sertoli cell extra cellular matrix. Therefore there would be accumulation of IGF-1, IGFBP-3, collagen, etc. affecting cell activities and integrity with increased network of fibrous tissue and the disappearance of the lumen. Abnormal deposition of collagen and proteoglycan within tissues of extra-cellular matrix components results in fibrosis, which may alter irreversibly the function of the involved organ or tissues and cells. The dysfunction will affect testicular spermatogenesis and steroidogenesis as both Leydig and Sertoli cells are affected. The lesion of interstitial cells, Leydig and peritubular cells will affect the action of LH that is localized in these cells Also, follicules stimulating hormone (FSH) activity will be affected with the disruption of Sertoli cells. At Days 7, 14 and 21 (Table 3) there was a significant reduction in cytochrome p450 level ($p<0.001$) compared to control. Cytochrome p450 is involved in several steps of the testicular testosterone biosynthesis. There is a close relationship between plasma level of LH and the activity of microsomal cytochrome p450 (Lee *et al.,* 1980). This showed that inhibition at Day 1, 14, and 21 and the corresponding reduction of the LH may be a direct consequence of chloroquine effect either by DNA intercalation of the gene that is necessary for various protein expression or by disruption of cell morphology. The cytochrome b5 contributes to testosterone biosynthesis in rat testicular microsomes (Ishii-Ohba Maisumara *et al.,* 1984). However at day 14 cytochrome b5 level was not significantly altered, and at day 21, there were significant ($p<0.001$) reduction of cytochrome p450, cytochrome b5 and NADPH cytochrome c reductase activities. Disruption of Leydig cells will affect the status of LH which regulates testicular cytochrome p450 and thus the significantly diminished cytochrome p450 levels observed in Days 7, 14 and 21 ($p<0.001$).

Table 2. Effect of daily chloroquine treatment on weekly of circulating plasma testosterone and plasma lutcinising hormone level.

Period Schedule	Testosterone nmol/l	Luteinizing Hormone (ng/ml plasma)
I. Control	8.45± 2.05	12.48±1.40
II. 7 Day		
ii Chloroquine (10mgkg^{-1} ip)	4.95 ± 1.42*$^{\phi}$	4.62 ± 0.6*$^{\phi}$
ii Control (Normal Saline 10mlkg^{-1} ip)	8.95 ± 1.91	11.95 ± 1.00
III. 14 day		
iii Chloroquine (10mgkg^{-1}, ip)	2.02 ± 0.05***$^{\phi\phi}$	2.45±0.95**$^{\phi\phi}$
iii. Control (Normal Saline 10mlkg^{-1} ip)	8.00 ± 01.94	11.50± 1.65
IV. 21 day		
iv. Chloroquine (10mgkg^{-1} ip)	1.83 ±0.10**$^{\phi\phi}$	1.05± 0.03**$^{\phi\phi}$
iv. Control (Normal Saline, 10mlkg^{-1} ip)	8.98 ± 1.97	11.85 ± 2.00

Data are means ± SE (N = 6). Each animal testosterone/LH assays were in triplicates. Statistical significance relative to control 1 (that is rat without normal saline administration) **P<0.001 and $^{\phi}$p<0.01 for within controls.

*P<0.01 $^{\phi\phi}$p<0.001 (control) 1

The effects on the proteins, cytochrome p450, cytochrome b5 and NADPH cytochrome c reductase may be due to an indirect effect of chloroquine – DNA intercalation that may diminish protein synthesis and disrupt enzyme activities.

The study has shown the risk factor that may be precipitated in sensitive patients during chronic chloroquine treatment. DNA intercalation, avid tissue binding, lysosomotropism and inhibition of receptor mediated endocytosis will culminate in the accumulation of collagen and the upgraded effects of IGF –1 and IGFBP –3 activities in Sertoli cells and somniferous tubules will cause fibrosis. Spermatogenesis and testosterone biosynthesis will be affected and so will be the fertility status. Whether there would be reversal of cessation of the endocrine problem of spermatogenesis and testosterone biosynthesis after stoppage of chloroquine ingestion requires further studies. The frequency of malaria attack and the ensuing chloroquine therapy requires additional studies on tissues of sexual organs as this may expose the infertility conundrum may be present in malaria areas.

Table 3. Effect of daily chloroquine treatment on cytochrome P. 450, cytochrome b5 and NADPH cytochrome C reductase in male rats testicular microsomes.

Treatment Period	Cytochrome P.450 (n mol/mg protein)	Cytochrome b5 (n mol/mg protein)	NADPH Cytochrome C reducatase (nmol/mim/mg protein)
Control (Normal Saline 10m kg^{-1} ip)	0.055 ± 0.001	0.0029 ± 0.002	10.66 ± 40
Day 7 Chloroquine (10mg /kg, ip)	0.044 ± 0.002*	0.032 ± 0.001	11.68± 0.80
Day 14 Chloroquine (10mg /kg, ip)	0.021 ± 0.001**	0.030 ± 0.002	8.02± 0.08
Day 21 Chloroquine (10mg /kg, ip)	0.015 ± 0.002**	0.016 ± 0.001**	4.02±15**

Values are expressed as mean ± SE, p* < 0.05 {(N = 6 samples) each samples consists of pooled tissues from 4 rats} and **P <0.001 are significantly compared to control. Chloroquine was administered as per the number of days as a above.

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