Afr J Med med Sci (1998) 27, 101-106

Sterological estimation of seminiferous tubular dysfunction in chloroquine treated rats

Abayomi O, Okanlawon and Oladapo A. Ashiru
Department of Anatomy, College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria.

Summary
We have used the simple-point sampling of linear intercept lengths and the optical dissector method to describe the effects of chloroquine on rat testicular morphology. The rats were injected intraperitoneally with chloroquine phospahate (CQ), an antimalaria and amphiphilic drug known to induce generalized lipidosis. The result showed that CQ (10 mg/kg/day) treatment for 7 weeks significantly reduced (i) the weight of the testis, (ii) the daily sperm production count, (iii) the total spermatoid count per testis and (iv) the star volume of the seminiferous tubules. There was however an increase in the total spermatocyte count per testis. Together, these findings suggest that disruption of spermatogenesis accompanied a decrease in tubular size in CQ treated rats.

Keywords: *Chloroquine, Seminiferous tubules, Disector-sample, Point-sample intercept.*

Résumé
Nous avons utilisé le poitn d'interception et al methode de dissection optique pour décrire les effets de la chloroquine sur la morphologie testiculaire du rat. Les rats ont été injecte intraperitonalement avec la chloroquine phosphate (CQ), un anti paludique et medicment amphiphilique connut pour son induction de lipidose generalisé. Les resultats montrent que le traitement à la CQ (10 mg/kg/jour) pendant 7 semaines a reduite de maiere significative (I) le poids des testucles, (ii) le compte de lar production journaliere de spermatozoides, (iii) le compte total de spermatozoide par testicule et, (iv) le volume des tubules sminiferes. Il ya cependant eu une augmention dans le compte total de spermatocytes par testcules. Toutes ces observations suggeretn que le traitement des rats par la CQ est accompagne par une reduction de la taille des tubules seminiferes et une perturbation de la spermatogenese.

Introduction

The effects of cationic amphiphilic drugs on various organs have been the subject of several rigorous investigations in the past [1-3]. Presently, over 30 cationic amphiphilic drugs (including CQ) induce the formation of concentric membranous structures (myeloid bodies) within cells when administered to intact organisms [4]. A number of comprehensive reviews have appeared on the toxicological implication of these structures on cellular, and tissue functions in the lungs, kidney, and eyes [1, 4-6]. Although previous histological, histochemical, and biochemical observations following repeated administration of CQ indicated increased adrenal cortex activity [7], to date, less information exists on the toxicological implication on the testis in animal experiments.

CQ produces a variety of biological effects in addition to its antimalaria action. It accumulates in lysosomes but does not cross the lysosomal membrane easily due to the protonation of the CQ moiety in the acidic cellular compartment [8]. In humans, the toxicity and side effects are minimal at the recommended dose (25 mg CQ base per kg body weight) in the treatment of malaria. However, in the treatment of various connective tissue disease, large doses (500 mg daily) and prolonged therapy (24 months) with CQ is necessary to achieve its anti-inflamatory effect with occurrence of side-effects normally not encountered during malaria chemotherapy [9].

Earlier studies from this laboratory have reported seminiferous tubular changes from measurements of tubular diameter, tubular epithelial height, number of spermatocytes per testis, and tubular total surface area following CQ (40 mg/kg/day, 5 days a week for 16 weeks) administration [10,11]. With respect to topology of the seminiferous tubules, it is difficult to extrapolate from these 2-dimensional measurements to 3-dimensional statements. Furthermore, a statement such as a decrease in tubular diameter is not strictly correct, although, it may be a useful index, because this assumes seminiferous tubules as isolated profiles in two dimensions which of course is not the case [12]. For qualitative determination of the effect of CQ on seminiferous tubules, we determined an unbiased 3-dimensional parameter (that is, the star volume); the total spermatocyte count per testis, and the total spematid count per testis. We provide evidence that CQ (10 mg/kg/day) administered over 7 weeks is deleterious to seminiferous tubular morphology.

Materials and methods

Animal and experimental design

Sprague-Dawley rats were used from an inbred colony maintained in the Department of Anatomy. They were housed under standard conditions (temperature 27-20 ^{0}C, 12l:12D photoperiodicity). They were given commercial rat food and had access to water *ad libitum*. Rats aged 7-8 weeks were divided into control and experimental groups. The experimental group received 10 mg/kg body weight CQ (Troge Medical GMBH, Hamburg, Germany) intraperitoneally, once a day between 1500 and 1600h, five days a week. The control animals received an equal volume of physiological saline. Throughout the experiment, the animals were weighed weekly. At the designated times (2, 4 and 7 weeks) four animals from each experimental group were euthanized by decapitation. The trunk blood was collected and serum was used for testosterone (T) determination by radioimmunoassay (RIA). We chose this extended period in order to assess the effect of CQ on general testicular morphology for one complete wave of spermatogenesis.

Hormone measurements

Serum T concentrations were determined in duplicate samples by a previously described RIA procedure [13]. The sensitivity of the assay was 10 pg/tube with intraassay and interassay coefficients of variation of 10.6 and 9.3, respectively.

Correspondence Dr Abayomi O Okanlawon, Department of Anatomy, College of Medicine, University of Lagos, Idi-Araba, Lagos

Tissue preparation for stereology
The testes from four rats from each of the two groups were prefixed (with Bouin's solution) by whole-body perfusion through the left ventricle [14]. Following perfusion, the testes were removed, weighted, and volume determined by the fluid displacement method [15]. They were then cut into three slices perpendicular to the long axis of the testis, and further fixed in fresh fixative for two hours. Each slice was oriented on a small piece of paper as previously described [16], embedded in paraffin wax, and entirely cut into slabs perpendicular to the long axis of the testis. The rationale for making slabs was that the tops and bottoms of the tissue slabs are easily differentiated from the sides, allowing blocks to be properly oriented at the time of embedding. The set of sections consisted of successive series of 20 serial sections of nominal thickness T = 25 um, followed by 10 thinner sections of nominal thickness T = 5 µm. Every 5th section from both section thickness was mounted and stained with Haematoxyline and Eosin (H&E). In order to relate the data obtained on testicular section to fresh testis, tissue shrinkage was determined according to the method of Wing and Christensen [17], and a correction factor of 1.20 applied to volume analysis.

Daily sperm production
In five additional animals per group, the testes were extirpated, weighed, and subjected to determination of daily sperm production (DSP) [18]. Briefly the testes were decapsulated and the seminiferous tubules were weighed. Seminiferous tubules were homogenized in 50 ml 0.154M NaCl + 0.5% (v/v) triton X-100, and 2% sodium azide. The concentration of homogenization-resistant elongated spermatid in the resultant solution was determined and DSP (sperm x 10^6/g/day) was calculated for each testis.

Tissue preparation for histology
In three additional animals per group, after an initial flush with physiological saline [13], the testes were perfusion-fixed with 5% glutaraldehyde in 0.2% collidine buffer (pH = 7.4) at room temperature for 30 – 45 minutes. Post-fixation was carried out in 2% osmium tetroxide containing 3% potassium ferrocyanide for two hours and embedded in Epon-araldite resin. The testes were then sectioned (1-2 um thick), and stained with toluidine blue for light microscopy.

Stereology
The star volume of the seminiferous tubules. ($\overline{Vv^*}$)
The star volume of an object is defined as the mean volume of all parts of the object that can be seen unobscured along uninterrupted straight lines in all directions from a particular point [19]. It owes its name to the fact that the structural characteristic it reports is clearly a volume and in a very complex structure like the seminiferous tubules [12], it is the only way to obtain a measure of size with a strict mathematical definition. The star volume of the seminiferous tubules (Vv^*) was estimated in an unbiased manner following the method described by Cruz-Orive and Weibel [20]. With the aid of a projecting microscope, with a 45^0 single mirror, the vertical sections of 5 um thickness were projected onto a white wall at a final magnification of x175. Using the L_0-rule, we measured only those intercepts whose sampling point hit a tubule and lies inside the frame and the class mark was determined. An unbiased estimator of the volume of the seminiferous tubule is:

$$\text{Est. } \overline{Vv^*}\text{ (tubule)} = \frac{\lambda}{3} \times \overline{l_0^3}$$

Where l_0 = (class mark) x (observed frequency in that class) / Observed frequencies.

To convert the mean class number of the observation to a mean value on the volumetric scale, taking the magnification x 175, and the ruler construction (15 classes = 3cm) into account, the arbitrary ruler unit used here equals 11.43 um^3.

$$\text{i.e., } Vv^* = \frac{\lambda}{3} \times \overline{l_0^3} \times 11.43\ um^3$$

Determination of total number of spermatocytes
The total number of spermatocytes per testis was estimated by the dissector principle applied to two thin optical sections inside the 25 um thick section [21,22]. A review of literature shows that the dissector method is not very common, but is known to be a good enumerator of 3-dimensional concepts with respect to the testis [11, 23-25]. Briefly, a special frame called the dissector is employed. The dissector is essentially a frame with two contiguous sides composed of dashed lines, and two contiguous sides composed of solid lines [22]. This frame determines what is counted. Every nuclear profile of germ cell in the box is counted except those intersecting the solid lines. Using an oil immersion optics with x 100 oil immersion lens, we focused down about 6 um to avoid distortion and uneveness of the section surface. AL germ cell nuclei seen in focus in this look-up plane were disregarded. The dissector frame (26 um by 17 um) was placed on this look-up plane. Then we moved the focus down in steps of 3 um for a distance of 9 um, counting all germ cell nuclei clearly focused in the dissector, and the total number of spermatocytes was estimated by the formula (2.1) in Gundersen [21]. During the germ cell number count, both optical sections were chosen well inside the physical section.

Statistical analysis
All statistical calculations were carried out using the Statistical Analysis (SAS version 6.03). A one-way analysis of variance (ANOVA) was used. Statistical significance was set at $P < 0.05$.

Results
The pre-treatment weights were similar in both control and experimental rats as the animals were aged-matched. During the duration of treatment, all animals steadily gained weight and the rate of weight gain was not significantly different among treatment groups. By the 4^{th} week of CQ administration, the absolute testicular weight of the experimental animals was essentially the same as that of the controls. Beginning from week 4, the absolute testicular weights of the CQ-treated animals decreased steadily; this decrease persisted throughout the remainder of the experiment.

Hormone levels
Serum concentrations of T declined markedly to 10% of control values after 4 weeks of CQ administration (1.9 ± 0.5 mg/ml v 0.19 ± 0.08 mg/ml). Further CQ administration beyond the 4^{th} week till the 7^{th} week did not significantly reduce serum levels of T (0.19 ± 0.08 mg/ml v 0.21 ± 0.07 mg/ml).

Daily sperm production (DSP)

Treatment of rats with CQ for 4 weeks did not alter the (DSP) when compared to intact control (Table 1). However in rats that were treated for 7 weeks, there was a significant reduction in DSP (> 0.50%) when compared to the control.

Table 1: Effect of CQ on testis weight, and daily sperm production (DSP) at the end of the 2nd, 4th and 7th week after commencement of treatment

		Testis	Weight (g)	DSP (sp x 106 /g/day)	
Week	n	Control	CQ	Control	CQ
2	5	1.49 ± 0.3a	1.48 ± 0.1	18.9 ± 2.2	17.1 ± 1.5
4	5	1.83 ± 0.1	1.81 ± 0.1	20.3 ± 1.4	18.6 ± 1.7
7	5	2.01 ± 0.2	1.63 ± 0.1b	21.0 ± 1.8	10.2 ± 0.6b

Control = Normal saline injected rats
CQ = CQ injected rats
n = Number of rats
a = mean ± SE
b = $P < 0.05$

Histological evaluation of the testis

Testicular tissue used for this study was well preserved for qualitative and quantitative morphological analysis. The appearance of the seminiferous tubules of control rats is shown in Fig. 1. The seminiferous tubules of rats treated with CQ for 4 weeks (Fig. 2) showed some degenerating cells with dissolution of the nuclear and cytoplasmic contents including the plasmalemma in the basal portion of some of the seminiferous tubules and spermatid sloughing. Although some of these spermatid were found in various phases of degeneration, many appears to possess normal morphology. These tubular effects were very pronounced in the tubules of those treated for 7 weeks and leydig cell atrophy was also very pronounced (Fig. 3). Despite the changes in the seminiferous tubules, the tunica propria did not appear thickened or hyalinised but remained as a delicate layer.

Fig. 1. Light micrograph of a control rat testis fixed by perusion and embedded in epoxy-resin (x 1000). Figure 1A shows the typical components of the interstitium consisting of leydig cells (L), blood vessels (V), and macrophages (M). Leydig cells are distinguished by their characteristic nucleus and dark cytoplasm. The seminiferous tubule and interstitium were well fixed. Sertoli cell (S) and pachytene spermatocytes (P) are indicated in Figure 1B

Sterological measurements

The star volume of the seminiferous tubules decreased from 270 um in the control to 90 um in CQ-treated rats. This decrease in star volume was independent of the duration of treatment. However, there was a significant increase in the total number of spermatocytes per testis, with accompanied decrease in total spermatid count per testis. These changes occurred in tubules with a substantial reduction in tubular volume (Table 2).

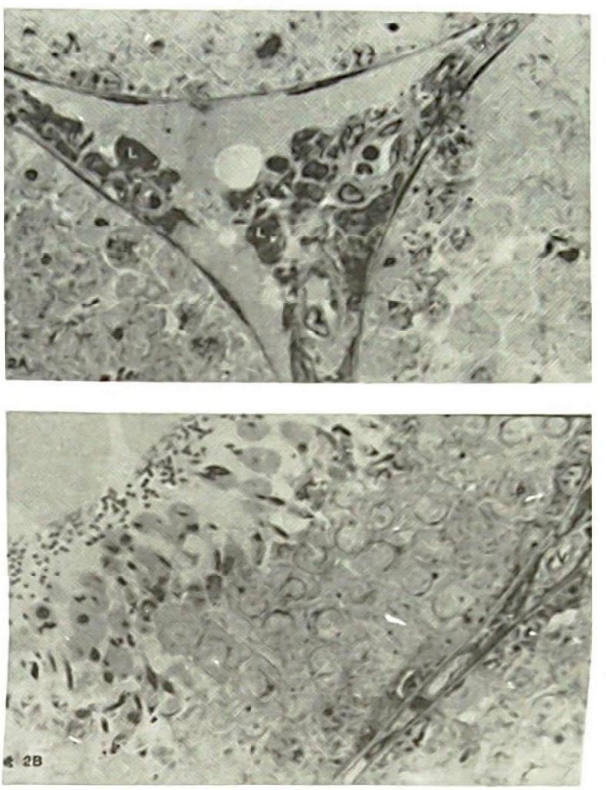

Fig 2. Light micrograph of rat testis treated with CQ for 4 weeks showing much less Leydig cell (L) cytoplasm surrounding the nucleus in Figure 2A and in the adjacent seminiferous tubules can be seen pachytene spermagonia (P). Figure 2B shows dissolution of germ cell cytoplams in the basal portion of the cytoplasm.

Table 2: Effect of CQ on star volume of seminiferous tubules ($\overline{Vv}$*), total spermatocytes count/testis (SP) and total spermatid count/testis (ST) at the end of the 7th week.

Group	Vv (um^3)	SP (x 10^6)	ST (x 10^6)
Control	270.4 ± 10.2a	120.0 ± 10.5	100.4 ± 7.0
2 weeks (CQ)	99.8 ± 8.0b	844.0 ± 70.9c	56.7 ± 6.1
4 weeks (CQ)	95.3 ± 10.1b	899.9 ± 40.5c	55.2 ± 9.0
7 weeks (CQ)	90.7 ± 5.0b	1090.1 ± 100.0c	45.8 ± 8.6b

Control = Normal saline injected rats.
CQ = CQ injected rats.
a = mean ± SD
b = $P < 0.05$
c = $P < 0.005$

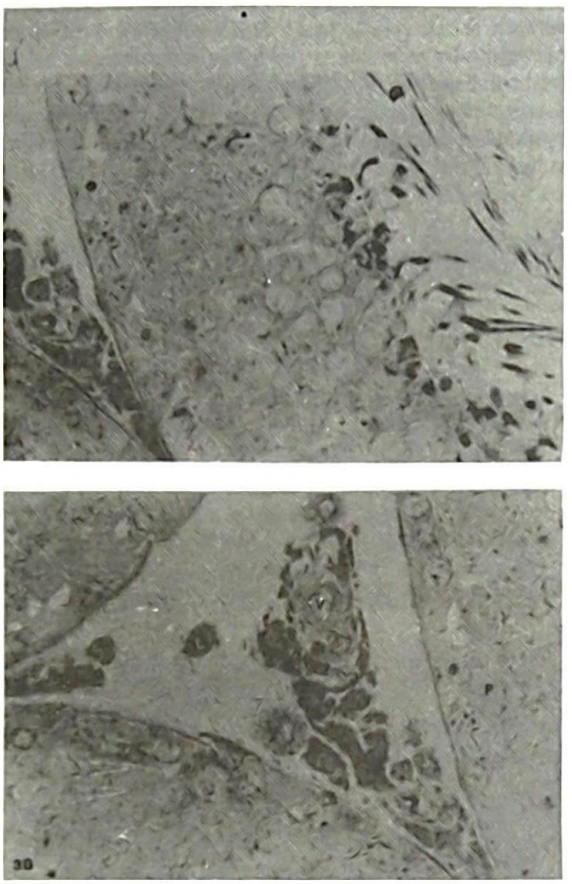

Fig. 3: Shows further reduction in Leydig cell cytoplasm (Figure 3A), and dissolution of germ cell cytoplasm (Figure 3B) in testis of rats treated with CQ for 7 weeks.

Discussion

Methodology

The point-sample intercept as described by Gundersen and Jensen [22] provides a new approach for an unbiased, and efficient estimation of the star volume from only one section (e.g., using independent sections). Previous methods for estimating the average volume of tubules from tissue sections essentially involved mathematical models in which tubules were assumed to be a round tube with a radius, a length, and a basement membrane surface area [17]. The point-sampling of linear intercept method circumvents this concept with complete absence of shape assumption [19,22]. Also, our data have been corrected for the volume changes that take place during fixation and processing for light microscopy, thereby extrapolating values to what might have been present in the fresh tissue.

Without the aid of a microcator, or a confocal scanning light microscope [21,22], the dissector height was measured imprecisely by the fine focus of the microscope. Notwithstanding, this bias is unlikely to be different in the experiment groups, and should not affect our conclusion.

We have used a stereological technique to evaluate the star volume and total number of spermatocytes within the testis regardless of the stage of the seminiferous cycle. This is in contrast to other studies that have limited their evaluation of a specific stage of the cycle, and are usually for short duration. We chose this approach because it has been observed that experimental manipulations, particularly those that result in the loss of spermatid stages may cause difficulty in identifying specific stages of the cycle [17,26].

Biological implication

This study provides quantitative data concerning the effect of CQ on the seminiferous epithelium. In our previous study we provided evidence that administration of very high doses of CQ (40 mg/kg/day), five days a week for 16 weeks led to a significant reduction in the volume of the testis , seminiferous tubules, and tubular lumens. The data from the present study confirm that concept, and provide information on the effect of clinical doses of CQ on the testis. Clearly CQ profoundly disrupts spermatogenesis with significant increase in the number of spermatocytes, accompanied by a decrease in elongated spermatids, and seminiferous tubular volume.

The role of the seminiferous tubules in the determination of the adult testis size has been widely discussed [27]. The important contributors are the germ cell population and the Sertoli cells. Unless other circumstances are at play to increase testis weight (tumours, edema, inflammation, etc.), there is a strong correlation between testis weight and the number of germ cell present in the testis [27]. The reduction in testicular weight and seminiferous tubular volume observed after CQ administration could result from a direct drug effect on germ cells, or could be secondary to a drug effect on Sertoli cells. This would necessitate (I) loss of germ cell and Sertoli cell population or (ii) germ cell and Sertoli cell cytoplasm or (iii) reduction in the luminal diameter of seminiferous tubules. Histologically, we found evidence for these three factors consistent with the marked reduction in luminal star volume. These observations suggest a decrease in Sertoli cell function as the presence of a size of a lumen is indicative of the function of the Sertoli cell that is responsible for the production of fluid into the seminiferous tubules [28]. In addition, this shrinkage occurred in tubules with significant increase in spermatocyte cell number. This suggests that the decrease in tubular size is probably as a result of the decrease in lumen size. A decrease in lumen size could indicate (I) enhanced absorption of luminal fluid, (ii) decrease fluid section into the lumen by the Sertoli cell, or (iii) increased pertibular myoid cell contractility [29]. Although we did not study alterations in seminiferous tubule fluid secretion, it is interesting to note that CQ has been shown to inhibit secretion of water, and electrolytes in rat intestinal perfusion loops *in vivo*, and chloride ion transport across isolated rabbit mucosa *in vitro* [30]. This antesecretory effect of CQ has been attributed to its capacity to inhibit phosphlipase activity [31,32]. In spite of the difficulty in providing detailed quantitative measurements of Sertoli cell function in the present study, it is still possible to approach the explanation for a decrease in luminal size. One approach is to focus on event occuring during the process of spermatogenesis. An increase in the number of spermatocytes per testis accompanied by decrease in the number of spermatid per testis would be consistent with either an increase in spermatocytosis or a decrease in the meiotic phase. An increase in spermatocytosis seems unlikely; a decrease in meiosis appears very probable, and would explain the decrease in the number of spermatids per testis. It is not possible to determine whether the effect of CQ is exerted at the first or second meiotic division, since the spermatocytes are not subdivided into primary and secondary in this analysis. It is important to note that the data on spermatids number did not involve stereological techniques, but were determined by

counting Triton-X 100-resistant (elongated spermatids) nuclei in a haemacytometer after testicular homogenization. Therefore, two different techniques were involved when comparing the number of spermatocytes and spermatids. Furthermore round spermatids were not determined in our study. Therefore, there is a gap of round spermatids in our data.

The disruption of the interstitium in CQ-treated rat is noteworthy. This suggests disruption of Leydig cell secretion and is consistent with a reduction in the serum levels of T. Although intratesticular T levels were not measured, the increase in number of spermatocytes per testis, coupled with a decrease in both spermatid count and DSP, provide an indirect evidence for a reduction in intratesticular level of T since it has been shown that T is essential for the completion of meiotic division [27,33,34]. It is therefore possible that the effects are seen in the seminiferous tubules Sertoli cell to androgen withdrawal. A direct drug effect on the Sertoli cell is also possible.

Clearly, these observations require additional studies to verify precisely the reason for the decrease in luminal size, the increase in spermatocyte count per testis, and the decrease in spermatid count in CQ treated rats. Certainly a reduction in the star volume of the seminiferous tubules would suggest changes in all cells (germ, myoid and Sertoli) that comprise the seminiferous tubules. In addition, changes in Sertoli cell function may also induce changes in germ cell, myoid cell and Leydig cell activity, as paracrine factors and cell-cell contact are believed to be important means of communication between cells in the testis.

We recognize that the duration used in this study was prolonged in comparison with the therapeutic dose schedule for malaria chemotherapy in humans; however quite apart from the general significance that the effects of CQ on seminiferous tubules have so far not been studied quantitatively, it should be remembered that small laboratory animals eliminate drugs at a higher rate than humans, thus requiring higher doses to obtain comparable blood levels.

In summary, this study demonstrates the CQ reduces the mean seminiferous tubular volume and increased the spermatocytes count per testis decreasing the spermatid count per testis. Taken together, these results suggest that CQ is deleterious to testicular morphology. The mechanism by which CQ might damage the seminiferous epithelium is uncertain. This is presently under investigation in our laboratory.

Acknowledgements

We thank Professor Martin Dym (Georgetown University Medical Centre, Washington DC, USA) for helpful discussion during the preparation of this manuscript, and Professor L.M. Cruz-Orive (University de Cantabria, Cantabria, Argentina) for the helpful comments with stereology. This study was supported by Rockefeller Foundation grant of OAA and AOO (GA PS 9105) and a Rockefeller Fellowship Grant of AOO.

References

1. Lullmann H, Lullmann-Rauch R, Wassermann O. Drug-induced phospholipidosis. Crit. Rev Toxicol 1975; 4: 185-218.
2. Reasor MJ, Kacew S, Thoma-Laurie DL. Effects of cationic amphiphilic drugs on the developing animal. In: Kacew S, Reasor MJ, eds. Toxicology and the new-born. Amsterdam: Elsevier, 1984; 64-84.
3. Lullmann R, Stoermer B. Generalized lipidosis in the new-born rats and guinea pigs induced during prenatal development by administration of amphiphilic drugs to pregnant animals. Virch Arch (Cell Biology) 1989; B39: 59-73.
4. Reasor MJ. A review of the biological and toxicologic implications of the induction of lysosomal lamellar bodies by drugs. Toxicol Appl Pharmacy 1989; 97: 47-56.
5. Hruban Z, Slesers A, Hopkins E. Drug-induced naturally occurring myeloid bodies. Lab Invest 1972; 27: 62-70/
6. Reasor MJ. Drug-induced lipidosis and the alveolar macrophage. Toxicol 1989; 20: 1-33.
7. Grundman M, Bayer A. Effects of chloroquine on adrenocortical function: II histological, histochemical and biochemical changes in the suprarenal glands of rats on long term administration of chloroquine. Arzneim Forsch 1976; 26: 2029-2035.
8. Okhuma S, Poole B. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc Natl Acad Sci 1978; 75: 3327-3331.
9. Estes ML, Ewing-Wilson D, Chow SM et al. Chloroquine neuromyopathy. Am J Med 1987; 82: 447-455.
10. Okanlawon AO, Ashiru OA. Effects of chloroquine on seminiferous tubular morphology in the rat. Discovery and Innovation 1992; 4: 65-69.
11. Okanlawon AO, Noronha CC, Ashiru OA. Increase in germ cell population and seminiferous tubular volume following chloroquine administration: stereological study using the disector method. Nig J Physiol Sci 1992; 8: 102-106.
12. Clermont Y, Huckins C. Microscopic anatomy of the sex cords and seminiferous tubules in growing and adult male albino rat. Am J anat 1961; 108: 79-98.
13. Falvo RE, Nabandov AV. Radioimmunoassay of peripheral plasma testosterone in males from eight species using a specific antibody without chromatography. Endocrin 1974; 95: 1466-1468.
14. Sprando RL. Perfusion of the rat testis through the heart using hepatin. In: Russell LD; Ettlin RA et al eds. Histological and Histopathological evaluation of the testis. Clearwater: Cache River Press, 1990: 277-280.
15. Weibel ER. Stereological Methods. New York: Academic Press, 1979.
16. Baddeley AJ, Gundersen HJG, Cruz-Orive LM. Estimation of surface area from vertical section. J Micros 1986; 142: 259-276.
17. Wing TY, Christensen AK. Morphometric studies onr at seminiferous tubules. Am J Anat 1982; 165: 13-25.
18. Robb GW, Amann RP, Killian CJ. Daily sperm production and epidermal sperm reserves of puberty and adult rats. J Reprod Fertil 1978; 54: 103-109.
19. Gundersen HJG, Jesen EB. Stereological estimation of the volume-weighted mean volume of arbitrary particle observed on random section. J Microsc 1985; 38: 127-142.
20. Cruz-Orive LM, Weibel ER. Recent stereological methods for cell biology: a brief survey Am J Physiol 1990; 258: L148-L156.
21. Gundersen HJG. Stereology of arbitrary particle: a review of unbiased number and size estimators and

the presentation of new ones; in memory of William R. Thompson. J Microsc 1986; 143: 3-45.

22. Gundersen HJG, Bagger P, Bendtsen TF, et al. The new stereological tools: Disector, Fractionator, Nucleator and Point-sampled intercepts and their use in pathological research and diagnosis. Acta Path Micro Immunol Scand. 1988; 96: 857-881.
23. Keeny DS, Mendis-Handagaa SMLC, Zirkin BR, Ewing LL. Effect of long term deprivation of LH on Leydig cell volume, Leydig cell number and steroidogenic capacity of the testis. Endocrin 1988; 1234: 2906-2915.
24. Mendis-Handagama SMLC, Ewing LL. Sources of error in the estimation of Leydig cell numbers in control and atrophied mammalian testes. J Microsc 1990; 159: 73-82.
25. Mendis-Handagama SMLC. Estimation of Leydig cell numbers of atrophied rat testes due to assumption of spherical nuclei. J Microsc 1992; 168: 25-32.
26. Sun Y-T, Wreford N, RobertsonD, deKrestser D. Quantitative cytological studies of spermatogenesis in intact and hpophysectomised ratsL: identification of androgen specific stages. Endorcrin 1990; 127: 1215-1223.
27. Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED. Histopathology of the testis. In: Russel LD, Ettlin RA et al eds. Histological and histopathological evaluation of the testis. Clearwater: Cache River Press 1990; 210-266.
28. Dym M, Raj HGM. Response of adult Sertoli cells and Leydig cells of depletion of LH and testosterone. Biol Reprod 1977; 17: 676-697.
29. Clermont Y. Contractile elements in the limiting membrane of the seminiferous tubules of the rat. Exp Cell Res 1958: 438-440.
30. Fogel R, Sharp GW, Donowitz M. Chloroquine stimulates absorption and inhibits secretion of ileal water and electrolytes. Am J Physiol 1982; 243: G117-G126.
31. Skaikh NA, Downar E, Butany J. Amiodarone-an-inhibitor of phospholipase activity: a comparative studdy of the inhibitory effects of amiodarone, chloroquine and chlorpromazine. Mol Cell Biochem 1987; 76: 163-172.
32. Liang YS, Peterson JW, Jackson CA, Reitmeyer JC. Chloroquine inhibition of cholera toxin. FEBS Lett. 1990; 275: 143-145.
33. Steinberger E. Hormonal control of mammalian spermatogenesis. Physiol Rev 1971; 51: 1-22.
34. Sun Y-T, Irby D, Robertson D, deKretser D. The effects of exogenously administered testosterone on spermatogenesis in intact and hypophysectomized rats. Endocrin 1989; 125: 1000-1010.
35. Bardin CW, Cheng CY, Musto NA, Gunselus GL. The Sertoli Cell. In: Knobil E. Neill J. et al. Eds. The Physiology of Reproduction. New York: Raven Press Ltd, 1988: 933-974.