Lipidemic Effect as a Manifestation of Chloroquine Retinotoxicity

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Summary
The effect of long-term treatment of chloroquine (CAS 54-05-7) (20 mg/kg body weight) on serum lipid components and its relation to the retinotoxic effect was studied in albino rats. Chloroquine was found to form lamellar lipoysome-like structures within the photoreceptor layer, as well as the pigment epithelium and neuroretinal layers.

Biochemically, hypolipidemia in the serum was observed mainly due to the decrease in phospholipid portion. It was hypothesized that due to the inhibition of the degradation process in the defective lipoysomes, the retinal cells were denied the re-use of their own phospholipids, and thereby resort to their uptake from the serum.

Zusammenfassung
Lipämische Wirkung als Manifestation der Retinotoxizität von Chloroquin

1. Introduction
Chloroquine (CAS 54-05-7)-induced morphological alterations observed in the retina of human beings and experimental animals are variable. In some earlier experimental studies, morphological alterations primarily affecting the retinal pigment epithelium have been observed [1, 2]. In others, however, the toxicity of the drug was found to affect primarily the morphology of the neuroretina and in particular its ganglion cells, whereas the morphology of the pigment epithelium was normal or deteriorated in the final stages only [3–6]. Others still reported that both the ganglion cells and the pigment epithelium were affected concomitantly [2, 7–10]. In the case of pigmented animals, chloroquine was found to affect the pigment epithelial cells by binding with melanin causing its accumulation [11]. In some reports, chloroquine treatment caused migration of melamin towards the inner retinal layers [2]. On the other hand, the evident retinotoxicity of chloroquine in albino animals and also in some pigmented animals was due to the accumulation of some lamellar bodies within the neuroretinal cells, especially the ganglion cells. These bodies were regarded as a hallmark of chloroquine-induced tissue lipidosis [12–14]. Yet, there are no reports in the literature pertaining to serum lipids in chloroquine toxicity and the metabolic changes accompanying this condition is still poorly understood and requires further efforts.

Accordingly, this study investigates the morphologic changes in the retinal tissue of albino rats chronically treated with chloroquine, and the variations in serum lipids accompanying it. The choice of albino species was based on the current consensus that melanin pigments by binding chloroquine, prevent the formation of lamellar bodies until its binding capacity is exceeded which may thus be the reason why the lamellar bodies are more frequently encountered in experiments on non-pigmented animals.

2. Materials and methods
2.1. Animals and experimental design
Fifty male albino rats weighing 150–200 g were used in this study. The animals were housed in separate cages and fed with standard laboratory chow and allowed drinking water ad libitum throughout the duration of the experiment. The animals were divided into three groups: the first group of 15 rats served as control. The second group of 20 rats was used for studying the toxic effects of chloroquine on the retina, while the third one of 15 rats was used to determine the effects of chloroquine on serum lipoid components.

2.2. Dosage
Rats of group two and three were injected with 20 mg/kg body weight chloroquine dihydroper (Sigma Chemical Company, St. Louis, MO, USA) dissolved in 150 mmol NaCl solution into the thigh muscle three times a week. Control group was injected with saline.

2.3. E. M. preparation
Eyes were enucleated from the rats under light ether anesthesia after 8, 16, 20 and 24 weeks of drug administration. The eyes were immediately fixed in 4% glutaraldehyde buffered at pH 7.3. Retinal tissue was then removed and further fixed in phos- phate buffered 1.3% osmium tetroxide (pH 7.3) for 1 h. The samples were then processed and embedded in araldite C52 according to the procedure of Glaser (15). Ultrathin sections were cut with an LKB ultratome, (LKB, Bromma, Sweden) stained with uranyl acetate and lead citrate according to the method of Reynolds [16] and examined with a Joel CX 100 TEM (transmission electron microscope) operated at 60 kV.

Retinal tissue from control animals was processed and exam- ined simultaneously for comparison.

2.4. Biochemical analyses
Blood samples from the retro-orbital venous plexus from lightly anesthetized rats [17] were drawn from the control group as well
as from the treated rats after 4, 8, 12 and 16 weeks of drug administration.

Serum total lipids were estimated by the Boehringer kit (Boehringer Mannheim GmbH, Mannheim, Germany) while cholesterol (HDL and LDL), phospholipids (HDL and LDL) levels and triglycerides were determined by the Bio Mérieux kits (Bio Mérieux, Marcy L’Etoile, France).

2.5. Statistical analysis

Student’s t-test was used to evaluate the significance of the data. Values of t at a level of p < 0.05 were considered significant.

3. Results

3.1. Ultrastructural observations

Light microscopic examinations revealed that the cytoplasm of both the pigment epithelial cells and the neuroretinal cells, especially the ganglion cell layer, contained dark stained bodies that increased in number with the prolongation of chloroquine treatment (Fig. 1 and 2). For the pigment epithelial cells, these bodies were located in the apical part thus increasing the thickness of the cells in the direction of the photoreceptors (Fig. 1). Vacuolization of the cytoplasm of the pigment epithelium and the ganglion cells was evident. Bruch’s membrane appeared thicker than normal. The photoreceptor cells appeared fragmented, disorganized and shortened. The other neuroretinal cells such as the cells of the inner nuclear layer and the ganglion cell layer appeared edematous.

At the ultrastructural level, the dark stained granules which accumulated within the pigment epithelium cells appeared as dense stained bodies (Fig. 3). An occasionally lamellar structure of some of these bodies was noted (Fig. 4). In addition, the apical microvilli of the pigment epithelium cells appeared ruptured and extended but ruffled and did not reach the photoreceptor outer segments in many parts (Fig. 3).

The photoreceptor outer segments appeared disrupted and shortened. Vacuoles and lamellar bodies were occasionally observed within the photoreceptor outer segments (Fig. 5).

In addition, the connections between the outer and inner segments of the photoreceptors were lost in several parts with the disappearance of the connecting cilium. The photoreceptor inner segments appeared edematous and balloon-like with peripherally pressed mitochondria (Fig. 6).

The dense granules which accumulated in the neuroretinal cells were more abundant in the ganglion cells but

![Figure 1: Semi-thin section of albino rat retina treated with chloroquine for 24 weeks showing the pigment epithelial layer loaded with dark stained granules and some small and large vacuoles. The outer segments of photoreceptors are ruptured in many parts and several gaps occur between the outer and inner photoreceptor segments. Bruch’s membrane appears thickened. Toluidine blue. × 750.](image1)

![Figure 2: Semi-thin section of albino rat retina treated with chloroquine for 24 weeks showing the inner layer. The ganglion cells show accumulation of dense granules in their cytoplasm. Giall cells show the presence of vacuoles (arrows). Toluidine blue. × 380.](image2)

![Figure 3: Electron micrograph of albino rat retina treated with chloroquine for 24 weeks showing the outer segments of the photoreceptors to be fragmented. Also an accumulation of dark stained bodies and presence of lipid droplets (arrow). Note the increased number of mitochondria migrating towards the apical cytoplasm. × 3150.](image3)

![Figure 4: Electron micrograph of albino rat retina treated with chloroquine for 24 weeks showing the dark bodies accumulated within the cytoplasm of the pigment epithelial cells. They consist of a homogenous or granular lamellae (A) surrounded by concentric dense lamellae (B). × 150 000.](image4)
Fig. 5: Electron micrograph of albino rat retina treated with chloroquine for 24 weeks showing dissolution in some parts of the outer segments of the photoreceptor cells and a lamellar body within one of them. An intact ellipsoid may be seen (arrow) × 4100.

Fig. 6: Electron micrograph of albino rat retina treated with chloroquine for 24 weeks showing the abnormal balloon-like ellipsoid part of the outer segments. These are ruptured in several parts (arrow) causing loss of connection with the outer segments. Note that the mitochondria are pressed towards the peripheries of the ellipsoid and fewer neurotubules. × 3150.

Fig. 7: Electron micrograph of one of the lamellar bodies accumulated within the cytoplasm of the ganglion cell. It consists of concentric whorls of laminated osmiophilic membranes. × 92000.

Fig. 8: Electron micrograph of albino rat retina treated with chloroquine for 24 weeks showing the ellipsoid of the inner photoreceptor segments. Note the ruptured ellipsoid (arrow) and lesser amount of neurotubules. × 11600.

Fig. 9: Electron micrograph of the albino rat retina treated with chloroquine for 24 weeks showing the inner plexiform layer and the ganglion cell layer. Note the variety of the dark stained bodies accumulated within the cytoplasm of the ganglion cell layer and the great number of vacuoles. × 1650.

were also found in the photoreceptor cells and cells of the inner nuclear layer and were usually formed of concentric lamellae (Fig. 7). Neurotubules were fewer and appeared ill-defined in the myoid and ellipsoid parts as compared to control sections (Fig. 6 and Fig. 8). The latter was also observed in the processes of the neuroretinal cells in the plexiform layers (Fig. 9).

3.2. Biochemical results
An insignificant drop in serum total lipids, triglycerides, cholesterol, HDL- and LDL-cholesterol levels was noticed after 4 weeks of chloroquine treatment in comparison to normal values, with the exception of phospholipids which showed a significant decrease. The significant change in serum phospholipids evident after 12 and 16 weeks of chloroquine treatment was due to the significant decrease in low density lipoproteins in the treated rats, in addition to the drop of HDL-phospholipids in comparison to normal (Table 1).
<table>
<thead>
<tr>
<th>Rat</th>
<th>Test</th>
<th>Total lipids</th>
<th>Triglycerides</th>
<th>Cholesterol</th>
<th>HDL-cholesterol</th>
<th>LDL-cholesterol</th>
<th>Phospholipids</th>
<th>HDL-phospholipids</th>
<th>LDL-phospholipids</th>
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<tr>
<td>Control Mean S.D.</td>
<td>277</td>
<td>52.0</td>
<td>80.0</td>
<td>59.0</td>
<td>15.0</td>
<td>126.0</td>
<td>90.0</td>
<td>19.0</td>
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<tr>
<td>4 weeks Mean S.D.</td>
<td>316.0</td>
<td>35.0</td>
<td>67.0</td>
<td>44.6</td>
<td>15.0</td>
<td>98.9</td>
<td>73.0</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td>8 weeks Mean S.D.</td>
<td>208.0</td>
<td>18.0</td>
<td>75.0</td>
<td>32.0</td>
<td>14.6</td>
<td>100.0</td>
<td>90.0</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>12 weeks Mean S.D.</td>
<td>224.0</td>
<td>46.16</td>
<td>78.9</td>
<td>55.16</td>
<td>17.90</td>
<td>96.9</td>
<td>78.9</td>
<td>10.6</td>
<td></td>
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<tr>
<td>16 weeks Mean S.D.</td>
<td>216.0</td>
<td>40.00</td>
<td>72.16</td>
<td>54.30</td>
<td>16.33</td>
<td>94.9</td>
<td>79.6</td>
<td>11.23</td>
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4. Discussion

The present study confirms that the chronic treatment of chloroquine to albino rats severely affects both the pigment epithelium layer and the neuroretinal cells. By the aid of both light and electron microscopy, the pigment epithelium cells were observed to increase in height and to contain unusual amounts of lysosomal-like structures in their apical cytoplasm. The thickening of the pigment epithelium cells is likely an adaptation to accommodate these accumulative structures.

The lysosomal origin of these structures was confirmed by localizing acid phosphatase activity within them [18]. The accumulation of such large amounts of lysosomes within the cytoplasm of the pigment epithelium [1, 2, 2–10] indicates an intensified rate of their formation and/or that they are not used up efficiently in the lytic process. Chloroquine has been described as a lysosomotropic agent [4, 16–21] whereby it enters the lysosomes and causes inactivation of the lysosomal hydrolases [22, 23]. Chloroquine, as a weak base, was found to accumulate in the acidic cellular compartment, thereby interfering in particular with the function of lysosomes and impairing the phagocytic activity of leukocytes [24]. Hence, although numerous lysosomes are formed in the cells, they may be defective in function.

Unusual gaps or spaces were observed between the pigment epithelium microvilli which, though extended, did not reach the tips of the photoreceptor outer segments. Chloroquine has also been described and used as a cytoskeletal inhibitor in various in vitro studies [25–27]. It is likely that by disrupting the actin filaments of the pigment epithelium cells, it leads to the interruption of the phagocytic process.

One of the main effects of chloroquine was directed towards the photoreceptors. The changes in the photoreceptor layer in the present investigation are in general agreement with the works of many scientists [1, 2, 7]. The observed edema in the inner segments may be a cause of the ruptured connections with the outer segments and diminished renewal of the latter. Due to edema, a hydrostatic pressure may be created leading to the swelling of the photoreceptor inner segments thereby pressurizing the mitochondria towards the peripheries of the cells, disrupting the neurotubules and rupturing the connections with the outer segments. This effect on the neurotubules may be intensified by the direct action of chloroquine which is known to cause disruption of microtubules [25, 28]. The neurotubules are known to function in the transport of secretory products and their diminished numbers in the inner segments will eventually affect the addition of newly synthesized membranes to the outer segments. In this way, the photoreceptor membranes are not renewed resulting in the diminished appearance of the outer segments and their inability to reach the microvilli of the pigment epithelium as described above.

Chloroquine retinotoxicity has been characterized by the accumulation of dark stained lamellar bodies within the pigment epithelium and the neuroretinal cells, especially the ganglion cells [3, 13, 29]. In the current study these lamellar bodies were also noted in the photoreceptors, an observation which has not been previously reported. It has been postulated that the pigment epithelium is spared the damage because of its capacity to bind the drug in an inactive form by the melanin granules [30] and only when this binding capacity has been exceeded that direct damage to the cells occurs. Hence, in albino animals, where melanin granules are absent from the pigment epithelium layer, the damage to the pigment epithelium and neuroretinal cells should be concomitant, as observed in this study.

Such accumulation of these lamellar bodies was regarded as a hallmark of chloroquine induced phospholipidosis [12, 14, 30]. Recently, an increase in the retinal phospholipids confined to the neuroretinal cells after chloroquine treatment was demonstrated biochemically [29]. The lipids were considered to originate from the continuous intracellular turnover of membranes and cell organelles [4, 13]. It is also suggested here that the lack of neurotubules in the axons of the neuroretinal cells lead to the accumulation of residues within the perikar-
yon, thereby providing additional biomembranes for turnover. The membranous discs of the photoreceptor outer segments, by their phospholipid rich content, may also interact with chloroquine to form lamellar inclusions, as seen in this study. Chloroquine was found to display high binding affinities to the amionic liposome phosphatidyl serine and interact by a lesser degree with phosphatidyl choline and ethanol amine [31]. It was also found that the lamellated inclusions formed in experimentally induced lipodystrophy by amphotericin B contained polar lipids in a lamellar phase [30].

In the present investigation, the disturbed lipid metabolism due to chloroquine treatment was found not to be confined only to the retina, but to affect serum lipids as well. Biochemically serum hyalopidemia was observed due primarily to a decrease in the phospholipid portion. This disturbed lipid metabolism may be explained by the accumulation of lamellar bodies due to the inhibition of the degradative process of the biomembranes that are catalyzed by the neuronneal cells for continuous turnover [14]. Accordingly, the retinal cells are denied the use of their own phospholipids, and thereby they resort to the uptake of more serum phospholipids causing a decline in their level.

The observed hyalopidemia may also be caused by the effects of chloroquine on other extra-ocular organs as the liver, adrenal cortex and the pancreas which are responsible for regulation of lipid metabolism. This suggestion is substantiated by previous studies where chloroquine treatment in rats cause an inhibition of the lysosomal phospholipase A & C that are present within the rat liver lysosomes resulting in an accumulation of phospholipids and cholesterol in their livers [32]. Also, it was suggested that chloroquine impairs the rate of mouse peritoneal idles lipid degradation in long-term experiments [33].

It is hypothesized that the retinotoxic effects of long-term treatment with chloroquine in albino rats is caused firstly by the accumulation of the drug in the retina. Secondly, chloroquine interacts with the biomembranes inhibiting the degradation of the lysosomes and becomes trapped in the lysosomes thereby acting as a lysosomotropic agent, and manifesting lipidic effects after prolonged use. Thirdly, chloroquine may exhibit disruptive effects to the cytoskeletal elements, consequently interfering with the physiological events in which they play a role such as secretion, phagocytosis, etc. To the author’s knowledge, this investigation is the first to note the implication of cytoskeletal effects of chloroquine in its retinopathy. However, immunohistochemical studies would be necessary to substantiate this finding.

In conclusion, in the long-term usage of chloroquine, control of treatment should be considered for each patient, and careful monitoring of the serum lipid pattern could be an accessible tool for weighing the risk-benefit effects of the drug.

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References