

Electroretinogram as an early detection of chloroquine retinal toxicity in pigmented rabbits

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Objective

Chloroquine (CQ) is a useful drug. It has less systemic hazards than many of the other medications used for immune diseases, but may cause retinopathy. The ophthalmologists do not discover the retinopathy till the appearance of bull's eye. The aim of the work is to try to use electroretinogram (ERG) and its b/a ratio in early detection of CQ retinopathy for pigmented rabbits chronically treated with CQ.

Materials and methods

Twenty adult pigmented rabbits (1.5–2 kg) were classified into four groups (five each): the first group served as control and the other three groups of rabbits were intramuscularly injected with CQ diphosphate (14 mg/kg body weight) three times/week for 50, 70, and 90 days, respectively. Each group is subjected to ERG recording, and then decapitation of animal and measurement of oxidant, antioxidant parameters, and histopathological examination in the retinal tissue were done.

Results

All results show no change by the 50th day after CQ administration. All the changes appear by the 70th day. The data indicated there is a deformation in ERG waves and significant decrease ($P < 0.05$) in b/a ratio. Significant increase ($P < 0.05$) in malondialdehyde level, and in the same context, significant decreases ($P < 0.05$) in superoxide dismutase, glutathione peroxidase, and catalase activity were seen. The histopathological changes are in the form of vacuolation in the pigment epithelium, destruction in the outer segments of the photoreceptor layer, and signs of karyolysis in some nuclei of outer nuclear layer.

Conclusion

Our data showed the possibility of using b/a ratio as an early indicator to CQ retinal toxicity in pigmented rabbits. It is also suggested that CQ increases the oxidative stress levels, so supplementation by antioxidants should be a part of the treatment regime.

Keywords:

b/a ratio, chloroquine, electroretinogram, lipid peroxidation, pigmented rabbits

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Introduction

Chloroquine (CQ) and its analog, hydroxychloroquine, are widely prescribed for rheumatoid arthritis, amebiasis disseminated, and lupus erythematosus. They were initially used for prophylaxis and treatment of malaria [1]. These medications may cause retinopathy with a predominant feature of advanced maculopathy. Different methods have been used to explore retinal dysfunction associated with CQ uses [2–5]. It is important to stress on the fact that CQ is a useful drug and has less systemic adverse effects than many of the other medications used for immune or inflammatory diseases. In spite of the abundance of researches on the CQ retinal toxicity, a controversy persists regarding whether the toxic effects of CQ act directly on the epithelium of retinal pigment or on ganglion cells or both. As indicated by past studies, some researchers suggested that the drug could interfere with the metabolism of the retinal pigment epithelium (RPE), and this may lead to secondary photoreceptor

degeneration [6–8]. Others recommended that the ganglion cell is the major site of toxicity [9–12]. The issue of CQ binding to melanin has been examined by numerous authors who suggested that the interaction of CQ with melanin may prompt retinal injury [13–15]. CQ retinopathy is irreversible, and growth of cellular damage may even increase after stopping of the drugs. When retinopathy is not known until the appearance of bull's eye, the disease can develop for years, with a subsequent loss of visual acuity. However, once retinopathy is recognized early, before RPE damage, there is only mild and limited progress after stopping the medication [16–18]. Thus, consistent follow-up cannot prevent damage, but if

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conducted aptly it enables the detection of toxicity before vision is significantly affected. Therefore, a very common method to avoid series retinal injury is to make sure patients that are taking the maximum amount of CQ had a screening [19]. Literature reports suggest that drugs such as CQ and primaquine used to treat malaria lead to oxidative stress, especially in erythrocytes [20,21], and induce lipid peroxidation in retina [22,23]. Bhattacharyya *et al.* [24] demonstrated that administration of CQ increases in NADPH that induced lipid peroxidation. Katewa and Katyare [25] have shown that prolonged use of antimalarials leads to severe impairment of energy metabolism and metabolic activity and alters the composition of mitochondrial lipids/phospholipids.

Although electroretinography (ERG) has been used in many studies to assess the functional retinal status during the usage of this drug [26], it does not recognize the initial phase of the retinal damage. ERG is a sensitive method of detecting changes in retina.

Our aim in this study is to try to use ERG and its b/a ratio in early detection of CQ retinopathy for pigmented rabbits chronically treated with CQ. Moreover, malondialdehyde (MDA) level and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities were measured in the retina to determine the oxidants and antioxidants parameters corresponding to retinal tissue. Finally, morphological changes in the retinal tissue were detected by histology after CQ treatment.

Materials and methods

Chloroquine used and dose

CQ diphosphate was obtained from the pharmacy as sachets produced by Sigma Chemical Co. (St Louis, Missouri, USA). Each sachet contains 100 g. CQ diphosphate was given in a dose of 14 mg/kg and dissolved in 0.2-ml saline by intramuscular injection three times per week [7]

Animals and the study design

Twenty adult pigmented rabbits weighing between 1.5 and 2 kg were used in this study. The animals were kept in the animal house of Research Institute of Ophthalmology, Giza, Egypt, under standard conditions of temperature and humidity (temperature: 22±2°C; humidity: 45–55%; light intensity: 300–400 lx). The animals were supplied proper pellet diet and water *ad libitum*. The experimental protocol was applying The

Association for Research in Vision and Ophthalmology statements for using animals in ophthalmic and vision research.

The animals were randomly divided into four groups (five each) as follows:

Group I: control group remained untreated.

Group II: received CQ diphosphate for 50 days.

Group III: received CQ diphosphate for 70 days.

Group IV: received CQ diphosphate for 90 days.

All groups of rabbits were subjected to ERG first and then decapitated. Their eyes were extracted to remove the retina for oxidants–antioxidants measurements and histology.

Recording of electroretinogram

ERG was recorded for the chinchilla rabbits in all groups using the [Neuro-ERG (Neurosoft Medical Diagnostics- Russia at the Research Institute of Ophthalmology, Giza-Egypt)]. ERG was recorded by using three skin electrodes: the active electrode is placed on the eyelid, and the reference and earth electrodes are placed on the two ears. To record full field ERG, frequency of 1 flash/second is used with no background intensity.

Oxidant and antioxidant measurements

At the end of the period of all groups, rabbits were decapitated and eyes were enucleated to remove the retina. Ten milligrams of homogenized retina was used for measurements of lipid peroxidation or MDA, SOD, GSH-Px, and CAT activity. MDA was measured calorimetrically in retina homogenate using assay kit of Sigma Aldrich Co. (Germany), according to the method of Dahle *et al.* [27]. SOD was measured by ELISA using an assay kit of Cell Biolabs Inc. (USA), according to the method of Durak *et al.* [28]. GSH-Px activity was measured by Cellular Activity Assay Kit of Sigma Aldrich Co. (Germany), according to method of Paglia and Valentine [29]. CAT activity was measured using assay kit of BioVision Inc. (Canada), according to the method of Aebi [30].

Histopathological examination of the retina

The eyes were enucleated, and intraocular injection of 0.1 M phosphate buffer (pH 7.3) containing 5.4% glucose and 4% glutaraldehyde was carried out for half an hour. The cornea and lens were removed, and the posterior segment of the eye containing the retina was cut into small pieces (about 1 µ) and then further fixed for another 8 h in fresh glutaraldehyde buffered solution. Then the specimens were fixed in

phosphate buffered osmium tetroxide for one hour at 4°C. They were washed for 1 h with several changes of phosphate buffer and dehydrated in cold alcohol series (50, 70, 80, 90, and 96%) for 10 min each. After dehydration, the specimens were then placed in propylene oxide for 10 min. Embedding of the retinal parts was carried out in Araldite mixture [31]. Finally, the specimens were embedded in rubber plates filled with freshly prepared araldite mixture and left at 60°C for 20 h. The blocks finally were polymerized at 70°C for 48 h. Blocks were sectioned by LKB ultra tome. Semithin sections were mounted on glass slide and stained with Toluidine blue. The slides are examined by a light microscope.

Statistical analysis

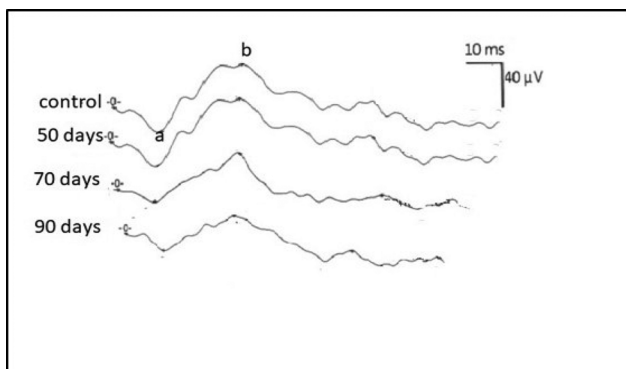
All results are recorded as mean±SD. For comparison between multiple groups, the analysis of variance procedure was used, where a commercially available software package (SPSS-11 for Windows; SPSS Inc., Chicago, Illinois, USA) was used, and the significance level was set at less than 0.05.

Results

Electrophysiological study

An ERG of a control and treated rabbits is shown in Fig. 1. The mean±SD of the amplitude of both a and b

Figure 1



Typical records of ERG for rabbits before and after chloroquine administration for all periods. ERG, electroretinogram.

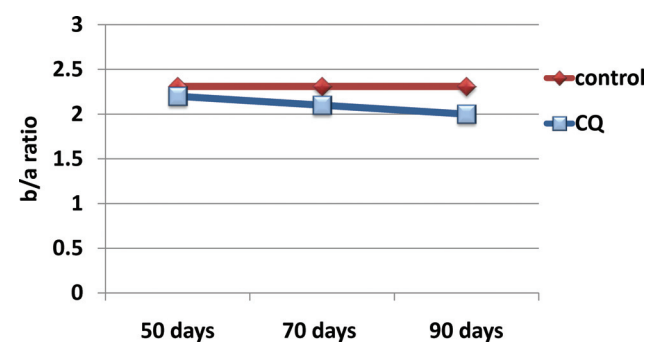
waves for the control and treated groups are stated in Table 1. For control, the amplitude and implicit time of a-wave were 26.1±0.5 μV and 13±0.26 ms, respectively, whereas those of the b-wave were 54±1.6 μV and 34 ±0.7 ms, respectively.

Amplitudes were measured from baseline to the lowest point of the negative peak for the a-wave and from the latter to the positive peak for the b-wave. The percentage difference [(control–treated)/control] % of ERG parameters showed no changes during the first 50 days. Slight changes appeared on the 70th day and significant changes ($P < 0.05$) on the 90th day. The changes were clear and noticeable. Significant increase ($P < 0.05$) of implicit time and significant decrease ($P < 0.05$) of amplitude for both a-wave and b-wave were observed. The data showed that a-wave is more affected than b-wave. It is clear from Table 1 that the decline of a-wave is faster than b-wave. The b/a ratio is also calculated which serves as a quantitative index and an examination to the vitality of the retina. In this work, 2.31±0.05 was found for control group. The ratio for the exposed groups showed a decrease throughout the period of the experiment, as is seen in Fig. 2.

Oxidant and antioxidant results

The results reported in Table 2 indicate the activities of MDA, SOD, GSH-Px, and CAT in retinal tissues. The table illustrated the control values of different

Figure 2



b/a ratio of ERG for rabbits before and after chloroquine administration for all periods. ERG, electroretinogram.

Table 1 a-wave and b-wave amplitudes (μV), their implicit time (ms), their ratio b/a, and percentage differences for treated groups compared with control

	a-wave		b-wave		b/a
	Implicit time (ms)	Amplitude a (μV)	Implicit time (ms)	Amplitude (μV)	
Control	13±0.26	26.1±0.5	34±0.7	54±1.6	2.31±0.05
50 days	12.9±0.25 0.8%	25.8±0.5 4%	34±0.5 0%	54±1.1 0%	2.2±0.04 5%
70 days	14.5±0.29 –12%	24.5±0.4 7.3%	36.2±0.46 –7%	52±1 4%	2.1±0.03 9%
90 days	16±0.4* –23%	22.4±0.4* 14.3%	38.2±0.5* –12.4%	50±0.7* 7.1%	2±0.03 13%

All data are expressed as mean±SD. %, [(control–treated)/control] %. *Statistical significant than control group at P value less than 0.05.

measured parameters, which were 5.13 ± 0.12 nmol/mg for MDA, 4.60 ± 0.18 U/mg for SOD, 135.23 ± 5 NU/mg for GSH-Px, and 4.69 ± 0.20 U/mg for CAT activity. The results indicated no significant changes in oxidant-antioxidant parameters owing to treatment with CQ for 50 and 70 days. However, after 90 days of treatment with CQ, there were significant increases ($P < 0.05$) in MDA value accompanying with significant decrease ($P < 0.05$) in SOD, GSH-Px, and CAT.

Histopathological examination

The structure of control group and histopathological changes of retina, after administration of CQ are shown in Fig. 3. Light microscopic examination of semithin sections for retina of control rabbit reveals normal histological appearance of the layers of retina

Table 2 Malondialdehyde level and superoxide dismutase, glutathione peroxidase, and catalase activities in retinal tissues for control rabbits and those treated with chloroquine after 50, 70, and 90 days

	MDA (nmol/mg)	SOD (U/mg)	GSH-Px (NU/mg)	CAT (U/mg)
Control	5.13 ± 0.12	4.60 ± 0.18	135.23 ± 5	4.69 ± 0.20
50 days	5 ± 0.24	5.02 ± 0.41	128.41 ± 4	4.74 ± 0.15
70 days	4.87 ± 0.30	5.42 ± 0.58	120.90 ± 7	4.35 ± 0.12
90 days	$7.30 \pm 0.20^*$	$6.90 \pm 0.13^*$	$95.74 \pm 3^*$	$2.26 \pm 0.15^*$

CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase. *Significant difference than control group at P value less than 0.05.

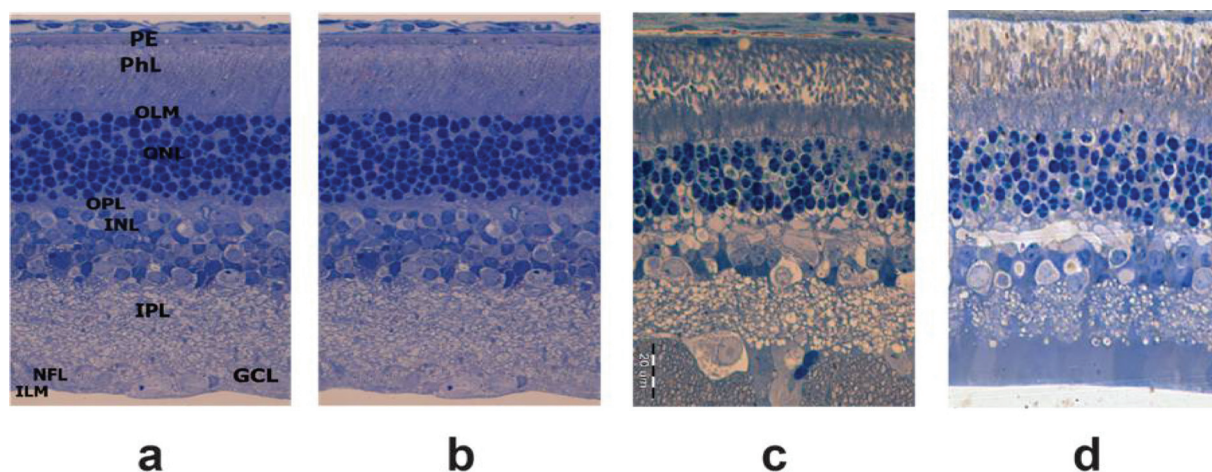
extending from outside toward the vitreous (Fig. 3a). These layers are arranged as follows: (a) pigment epithelium layer (PE), (b) photoreceptor (ph.), (c) outer limiting membrane (OLM), (d) outer nuclear (ONL), (e) outer plexiform (OPL), (f) inner nuclear (INL), (g) inner plexiform (IPL), (h) ganglion cells (GCL), (i) nerve fiber (NFL), and (j) inner limiting membrane (ILM).

Light microscopic examination of rabbit's retina that received CQ for 50 days showed no histopathological changes (Fig. 3b). There was a slight fragmentation in group III in the photoreceptor layer (Fig. 3c), and this change increased and became obviously in group IV, which received CQ for 90 days (Fig. 3d). The changes are in the form of vacuolation in pigment epithelium layer and distribution in the outer segments of photoreceptor layer. Moreover, some nuclei of outer nuclear layer were surrounded by a hallow clear area, whereas other showed signs of karyolysis. Edema in outer plexiform layer appeared. Some nuclei of inner nuclear layer showed signs of karyolysis after 90 days (Fig. 3c and d).

Discussion

In the current study, pigmented rabbits were preferred because of the similarity of their eye to the human eye [7]. A dose of 14 mg/kg thrice a week was given. This used dose is approximately 1.5 times that of the therapeutic dose used in the treatment of malaria disease; whereas the therapy of rheumatologic and

Figure 3



Histological examination of rabbit retina for all groups. (a) Control rabbit showing the different layers of retina. PE, pigment epithelium; PhL, receptor layer; OLM, outer limiting membrane; OPL, outer plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer; INM, inner limiting membrane (toluidine blue $\times 1250$). (b) Group II showing no change in all layers of retina of rabbits that received chloroquine for 50 days (toluidine blue $\times 1250$). (c) Group III (group that was administered with chloroquine for 70 days) shows there is a slight change in the photoreceptor layer of rabbit retina (toluidine blue $\times 500$). (d) Group IV (group that received chloroquine for 90 days) showing the fragmentation and more changes appear in the photoreceptor layer (toluidine blue $\times 500$).

dermatological diseases needs higher doses, which suggests rise in its risk. The current investigation showed that the ERG changes of rabbits started after 70 days and were evident within 90 days of CQ administration. The changes were in the form of statistically significant increase and decrease ($P \pm 0.05$) of implicit time and amplitude, respectively, for both a-wave and b-wave. The present finding agrees with Lezmi *et al.* [6].

ERG represents the electrical activity generated by neural and non-neuronal cells within the retina in response to a light stimulus. The a-wave is an early corneal-negative deflection, derived from the outer photoreceptor layer, and measures its activity. The b-wave is a corneal-positive deflection, derived from the inner retina, predominantly Muller and ON-bipolar cells [32]. The a-wave amplitude depends on the reliability of the photoreceptor, whereas the amplitude of b-wave depends on the a-wave and of the interactions between the a-wave and b-wave generators [33]. Our results agree with the suggestion of CQ interaction with RPE being the cause of secondary photoreceptor degeneration, that is clear in results of a-wave and b-wave and b/a ratio. CQ has an affinity for pigmented (melanin-containing) structures. Melanin is a free-radical stabilizer and might catch toxins, including retinotoxic medication. However, melanin binding could represent a mechanism for eliminating toxic agents from intracellular sites of damage. This may explain why ERG showed no changes during the first 50 days.

In animal studies, CQ exposure damaged inner and outer retina, but recent research proposes that inner retina is not injured [34,35], which agrees with our histopathological results (Fig. 3c and d). In clinical practice, the first harm is to the rods and cones, and as the outer nuclear layer degenerates, there is secondary disorder of the RPE [36]. So, this clarification may give a good idea about the resulted deformation in the ERG. It is noticed in the current study that, on the 70th day mark, certain alterations were observed; the b/a ratio decreased owing to decreasing both of a-wave and b-wave amplitude, and the a-wave rapid decrease was much more noticeable (7.3–14.3%) than the b-wave (4–7.1%) as it was much faster. The previously mentioned findings were between a 20-day interval (70–90 days).

The retina which is considered as neurosensory tissues has many unsaturated fatty acids and is thus highly sensitive to oxygen-free radicals. The measurement of lipid

peroxidation is a convenient method to monitor oxidative damage. The oxidative stress induced by CQ leads to extreme formation of free radicals and improved lipid peroxidation resulting in retinopathy. Thiobarbituric acid-reactive substances produced by lipid peroxidation can cause cross-linking and polymerization of membrane constituents. This can modify the natural membrane characteristics such as deformability, ion transport, and enzyme activity [37], and this is in containment with the results of oxidants–antioxidants parameters owing to treatment with CQ after 90 days.

SOD is a metalloprotein and is the first enzyme involved in the antioxidant enzyme by lowering the steady state level of O^{2-} . CAT is a heme protein, which catalyzes the decomposition of hydroperoxide to water and oxygen and thus protects the cell from oxidative damage by hydroperoxide and $OH^\bullet$. GSH-Px was an enzyme catalyze the reduction of hydroperoxide by glutathione [38]. In our study, decline in the activities of these enzymes in CQ-administered rabbits reveals that lipid peroxidation and oxidative stress are elicited by CQ intoxication. Lipid peroxidation in this tissue is extremely deleterious, because the ERG is produced by a series of differences in membrane ionic permeability. Correlating the previous data, it can be suggested that testing this b/a ratio could possibly alert to a problem in the retina early on, before any serious damage happens.

Conclusion

Our experimental data showed the possibility of using b/a ratio as an early indicator to CQ retinal toxicity in pigmented rabbits. As the ERG was mimicked on rabbits to those described in humans, we recommend to inform patients about the risk of ocular toxicity of CQ and their proper dose levels and the importance of regular follow-up. It is also suggested that supplementation of antioxidants should be in part of treatment plan for management of the retinal toxicity of CQ.

Declaration of interest

The author reports no conflicts of interest. The author alone is responsible for the content and writing of this paper.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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