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Chloroquine causes similar electroretinogram modifications, neuronal phospholipidosis and marked impairment of synaptic vesicle transport in Albino and Pigmented Rats

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ABSTRACT

Retinal toxicity of chloroquine has been known for several years, but the mechanism(s) of toxicity remain controversial; some author support the idea that the binding of chloroquine to melanin pigments in the retinal pigmented epithelium (RPE) play a major toxic role by concentrating the drug in the eye. In our study, 12 albinos Sprague-Dawley (SD) and 12 pigmented Brown Norway (BN) rats were treated orally for 3 months with chloroquine to compare functional and pathological findings. On Flash electroretinograms (ERG) performed in scotopic conditions, similar and progressive (time-dependent) delayed onset and decreased amplitudes of oscillatory potentials (from Day 71) and b-waves (on Day 92) were identified in both BN and SD rats. In both strains, identical morphological changes consisted of neuronal phospholipidosis associated with UV auto-fluorescence without evidence of retinal degeneration and gliosis; the RPE did not show any morphological lesions or autofluorescence. IHC analyses demonstrated a decrease in GABA expression in the inner nuclear layer. In addition, a marked accumulation of synaptic vesicles coupled with a marked disruption of neurofilaments in the optic nerve fibers was identified.

In conclusion, ERG observations were very similar to those described in humans. Comparable ERG modifications, histopathology and immunohistochemistry findings were observed in the retina of both rat strains suggesting that melanin pigment is unlikely involved. chloroquine-induced impairment of synaptic vesicle transport, likely related to disruption of neurofilaments was identified and non-previously reported. This new mechanism of toxicity may also be responsible for the burry vision described in humans chronically treated with chloroquine.

1. Introduction

Chloroquine toxicity remains a problem in many parts of the world where the drug is widely used for the treatment malaria, and chronic inflammatory diseases such as systemic lupus erythematosus and rheumatoid arthritis (Cooper and Magwere, 2008; Jover et al., 2012). More recently, it was demonstrated that Chloroquine also has potential for use as a chemosensitizer in cancer in conjunction with other conventional treatments or for inhibition cancer development and metastasis in vivo (Vezmar and Georges, 1998; Zhao et al., 2005; Savarino et al., 2006; Jiang et al., 2010).

Retinal toxicity of chloroquine/hydroxychloroquine has been extensively studied since its first description in 1959 (Hobbs et al., 1959). Recently, the American Academy of Ophthalmology published a manuscript with new recommendations on screening for

Abbreviations: BN, Brown Norway; ChAT, Choline-Acetyl-Transferase; ERG, Electroretinogram; GABA, Gamma Amino Butyric Acid; GC, Ganglion cell; GFAP, Glial Fibrillary acidic protein; HE, Hemalun & Eosin; IHC, Immunohistochemistry; IPL, Inner plexiform layer; INL, Inner nucler layer; mfERG, multifocal electroretinogram; OPs, Oscillatory Potentials; OPL, Outer plexiform layer; ONL, Outer nuclear layer; Pt, Pretest; RCL, Rod and cone Layer; RPE, Retinal pigmented epithelium; SD, Sprague Dawley; VIM, Vimentin.

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chloroquine retinopathy (Marmor et al., 2011). Indeed, while significant reversible or irreversible central visual loss associated with the drug is very rare, it is an important side effect that can warrant discontinuation of therapy (Jover et al., 2012). The clinical picture of chloroquine retinopathy is characterized by a paracentral visual field scotoma with associated parafoveal retinal pigment epithelium (RPE) atrophy, known as 'bull's eye' maculopathy (Marmor et al., 2011). Several studies have been conducted to examine treated patients by global electroretinogram (ERG) and multifocal electroretinogram (mfERG). In brief, these studies showed that mfERG can reliably detect retinal functional loss associated with chloroquine/hydroxychloroquine retinopathy. In some patients, the mfERG showed reduced ERG response amplitudes when other functional tests (e.g. visual acuity, color vision, central visual field) or morphologic examinations (e.g., ophthalmoscopy, fundus autofluorescene, fluorescein angiography) were normal (Kellner et al., 2006).

In animals, chronic exposure to chloroquine has been studied in many species including the rat, rabbit, mouse, pig, cat, and monkey (Leblanc et al., 1998). Due to its capacity to bind melanin pigment (Mars and Larsson, 1999; Ono et al., 2003; Yamada et al., 2011) it has been suggested that binding to melanin may result in concentration of the drug in ocular structures leading to an accumulation of the drug and thus the potential for increased toxicity. However a review of the literature on drugs binding to melanin, suggest an absence of a causal relationship between melanin binding and retinal toxicity (Leblanc et al., 1998).

To investigate and clarify the potential role of melanin pigment, albino Sprague Dawley (SD) and pigmented Brown Norway (BN) rats were orally treated with chloroquine for 3 months. ERG, histopathologic and IHC examinations of the retina were performed to assess both functional and morphological parameters in these two strains of rats.

2. Materials and methods

2.1. Animals and treatment

Twelve Sprague Dawley (SD, non-pigmented) and 12 Brown Norway (BN, pigmented) male rats from Charles River Laboratory (L'Arbresles France), 11 weeks old, were treated at 30 mg/kg/day from day 1 to day 49 then at 50 mg/kg/day from day 50 to day 92 (due to a lack of ERG response in the first period). Chloroquine diphosphate (salt form, Sigma) diluted in a vehicle (sterile water) was administrated by the oral route (5 mL/kg) for 92 days. Ten BN and 10 SD male control rats were daily administrated the vehicle only (5 mL/kg); these control rats were used for the histopathological analyses only.

Rats were paired in cages in a controlled environment (air renewing: 10–20 times/h, room temperature: 17–21 °C, relative humidity: 45–65%, circadian rhythm: 12 h).

Body weights were recorded every week during the study. Treated SD rats only presented with a mild decreased body weight gain and decreased final body weights when compared to controls. This was associated with decreased food consumption. BN rats were not affected. It was concluded that variations of body weights in SD did not affect the ERGs and pathological observations.

2.2. Electroretinography (ERG)

The objective of the ERG analysis was to evaluate the coupled functioning of the retina and retinal pigmented epithelium (RPE). Detailed ERG procedures were previously reported (Rosolen et al., 2005, 2008). Briefly, rats were kept in the dark for approximately 12 h to obtain maximum amplitudes and stable ERG parameters. Rats were then placed in a red light room sufficient for animal manipulation in preparation for ERG with minimal impact on rod photopigment activation (Hurley et al., 1977) and anesthetized (Imalgen® 50 mg/kg, Domitor® 0.1 mg/kg, intramuscular injection); induction of mydriasis was done using Mydriaticum®. A V8-9 version Visiosystem (Siem Bio-Médicale, Nîmes, France) was used to generate the flash stimuli as well as record (recording bandwidth: 0.1–300 Hz, 6 db per octave) and analyze the ERG responses. Monocular full field ERGs (RE) were evoked with the use of flash of light (source: xenon capacitive discharge) delivered in scotopic conditions (dark). The electroretinographic responses were recorded during 250 milliseconds. The cornea was kept hydrated by application of carbopol (Ocrygel®, TVM, France). The ERG was recorded with the active corneal electrode [(DTL fiber electrode, X-static® conductive nylon yarn, Sauquoit Industries, Scranton, PA, USA) that wind up a Genuine Grass 12 mm in diameter (Astro-Med inc West Warwick, USA)] placed onto the retinal surface and kept moist with the use of carbopol (see above), as previously described (Rosolen et al., 2004; Joly et al., 2006; Dorfman et al., 2009). This heavy gel also maintained the eyelids open. The reference electrode (disc electrode: Genuine Grass 6 mm in diameter, Astro-Med inc., West Warwick, USA) and ground electrode (acupuncture type: Europa Marco Polo, MPE 13-Biomed, Nîmes, France) were positioned in the mouth and subcutaneously in the tail, respectively.

For all animals and in all occasions, retinal function was assessed in scotopic conditions with the use of an average of 3–5 flashes of 15 increasing intensities (min intensity: $-3.99\ \log cds/m^2$; max intensity: $0.21\ \log cds/m^2$) delivered at 0.1 Hz temporal frequency, which enables the amplitude of the ERG to be plotted as a function of the intensity of the flash stimulus. This scotopic luminance-response (SLR) is generated in order to determine the V_{max} (maximum b-wave amplitude observed at the saturation point of luminance curve). Typically, the SLR is devoid of a-wave when intensities of the stimulation are within the scotopic range but yield an ERG identified as the scotopic mixed rod-cone response (including an a-wave) when the intensities of stimulation are within the photopic range. Amplitudes (from baseline to peak) and implicit time (time-to flash onset to-peak of the waves) of Oscillatory Potentials (Ops), a- and b-waves were recorded before treatment (pretest, Pt)) and on days 8, 22, 36, 71 and 92. A-wave amplitudes were measured from baseline to the a-wave trough, b-wave amplitudes were measured from the a-wave trough to the b-wave peak, and a- and b-wave implicit times were measured from the flash onset to the a-wave trough and the b-wave peak, respectively (Rosolen et al., 2005, 2008). The major OP waves (OP1, OP2, OP3 and OP4) were extracted from flash responses at V_{max} (see above) using a 100–300 Hz bandwidth electronic filter as showed in Fig. 1. Amplitudes were measured like b-wave parameters from trough to peak and implicit times from flash onset to OP peak.

A Student's paired *t*-test was performed to determine the statistical significance; as four measures were evaluated for the same ERG, a Bonferroni correction was used giving significant threshold at $p < 0.012$ instead of 0.05 for amplitudes and implicit times of "a" and "b" waves, and for OP1, OP2, OP3 and OP4.

All studies and the procedures of our laboratories complied with the French regulation (Decree 87–848 revised 2001) implementing European Directive 86/609 and European Convention ETS123. Furthermore, the studies were performed in accordance with the standards of the ILAR Guide and the Sanofi Charter on the Humane Care and Use of Laboratory Animals, including ethical review.

2.3. Histopathology and Immunohistochemistry

Animals were sacrificed by exsanguination under anesthesia at the end of the treatment period (24 weeks old). Eyes of rats were fixed in Davidson's solution (Sigma) for 48 h and routinely embedded in paraffin. Histological slides (4 μm in thickness) were produced and stained with hemalun–eosin. For immunohistochemistry, sections were placed onto pretreated glass slides (StarFrost). Slides were dewaxed and rehydrated in water, then used for standard immunohistochemical analyses. Each section was incubated with one of the primary antibodies shown in Table 3. The primary antibodies were detected using an adapted secondary biotinylated antibody and avidin–biotin–peroxidase complex (ABC) system (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine DAB (Zymed, San Francisco, CA, USA) as chromogens, which produce brown deposits. A slight counterstaining was done with aqueous hematoxylin. Nonspecific binding was controlled by omitting the primary antibody and using normal mouse serum (Zymed, San Francisco, CA, USA).

Morphological analyses were performed using "Image J" (NHI software) to evaluate the number of ganglion cells in a representative area of the retina (obj20, corresponding to 880 μm in length of the retina), the thickness of the rod and cone layer (3 measures per animal), and the thickness of the outer nuclear layer (ONL) (4 measures per rat) on representative fields of the retina.

A Mann-Whitley test was used on the means of each criterion, to evaluate the potential loss of neurons and/or rods and cones using Stata software; significant threshold at $p < 0.05$.

3. Results

3.1. ERGs

3.1.1. ERG morphology

For all animals and in all occasions, ERG morphology was normal with a negative a-wave (whenever recordable) and a positive b-wave, both discernible from the background noise (Fig. 1A, C). Similarly, the major Ops (OP1, OP2, OP3 and OP4) were discernible from the background noise (Fig. 1B, D). After approximately 12 h of dark-adaptation, b-wave amplitude responses in BN rats were more intense (up to 624 μV) when compared to SD (up to 361 μV) as noticed on the pretest values (Table 1). In both strains no ERG changes were observed on days 8, 22 and 36. The first statistically