Alternative method in experimental ERG for retinal toxicity

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We aimed to define a cost-effective and alternative method for experimental electroretinography (ERG) in rabbits. The trigger input port of data acquisition device was connected to output port of an unemployed EEG device. The exposure area of photic stimulator was firmly covered by Wratten neutral density filters with variable optical densities (ODs). Different optical transmissions were obtained by putting more than one filter over the other one. The illumination of the area at the level of rabbit eye was measured by a luminometer in photic stimulations. ERG was performed to the both eyes of three albino rabbits in scotopic and photopic conditions at the baseline. Intravitreal saline injections were performed in right eyes of the rabbits. ERG and ophthalmologic examination were repeated one week later. ERG responses were obtained by short-duration light stimuli with different strengths in scotopic (-2.69; -1.69; 0.00; 0.30; 0.69; 0.90; 1.10; 1.30; 1.69; 2.00 log stimulus energy (log cd.s/m²)) and in photopic conditions (1.3; 1.69; 2.0; 2.10; 2.30 log stimulus energy (log cd.s/m²)). Although minimal decays in amplitudes of a- and b- waves were detected after saline injection, there was no significant difference between baseline and after injection for the stimulus-response time of a- and b- waves (p>0.05). An unemployed EEG device can be effectively used for photic stimulation in experimental ERG in the studies of retinal toxicity.

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1. Introduction
Electroretinography (ERG) is a test that measures the physiological response of retina to light exposure in light and dark adapted state of the subject. ERG is generally needed for detecting of retinal toxicity or retinal function loss in experimental study (Huang et al., 2015).

Assessing retinal drug toxicity is becoming increasingly important and routine as different molecules are now developed for the treatment of retinal diseases and vascular disorders. In pharmacology and toxicology, ERG can be used to quantify the possible side effects of any systemic drugs or intraocular administrated agents (Huang et al., 2015).
used an unemployed electroencephalography (EEG) device for photic stimulation. EEG is electrophysiologic test that is used for diagnosis of some neurological disease, particularly for epilepsy and flash EEG is technically similar to ERG. It has photic stimulator and it amplifies weak electrical signals to analysis, and it is performed by light flashes with different frequencies and these frequencies can be adjusted by control panel over the device (Fylan et al., 1999).

Compact experimental set of ERG may not be easily available, especially for laboratory studies because of high cost. We combined and synchronized the photic stimulator of an unemployed EEG machine with a basic data acquisition device and we defined a cost-effective method to measure electrical response of retina for investigating the retinal toxicity in animals.

2. Experimental procedure

The Ondokuz Mayıs University Animal Care and Use Committee, Samsun, Turkey, approved all aspects of this investigation, and all experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology and European Union guidelines for the use of animals in research. Healthy female New Zealand white rabbits, weighing between 2.0 and 3.0 kg, were housed in separate cages. Three rabbits were maintained in a controlled environment with a 12-hour on/off light cycle, and food and water were administered ad libitum. All procedures were performed with these rabbits under anesthesia induced with an intramuscular injection of ketamine hydrochloride (35 mg/kg body weight; Ketalar 50 mg/ml; Pfizer; Istanbul, Turkey) and xylazine hydrochloride (5mg/kg bodyweight; Rompun 2%; Bayer, Germany).

Pupils were fully dilated by phenylephrine hydrochloride 2.5% (Mydfrine; Alcon Lab., Inc.; Texas, USA) and cyclopentolate hydrochloride 1% (Sikloplejin; Abdi İbrahim; İstanbul, Turkey) throughout the entire procedure in order to achieve a maximal retinal illumination. Pupil size was measured at the beginning and at the end of testing procedure. Eyelids were kept open by speculum during the examination and both corneas protected with a viscoelastic substance solution such as 1% methylcellulose in order to prevent corneal dehydration as well as to maintain a stable corneal potential. Electroretinographic waveforms were obtained from the eye simultaneously by positioning the active electrode (ERG-jet monopolar contact lens electrodes; Universo Plastique SA; Le Cret-Du-Locle, Switzerland) on the cornea. Reference electrode was placed at the junction of the ear and the temple whereas ground electrode was inserted subcutaneously in the interscapular zone. Rabbits were kept in fully dark room for dark adaptation at least 30 minutes and the duration for light adaptation of the rabbits was at least 15 minutes in the study.

Device set up

Corneal electrode was inserted to the port of negative pole and reference electrode was inserted to the port of positive pole and ground electrode to ground port on the bio-amplifier connector. The exposure area of photic stimulator was firmly covered by 15.3 cm x 3.8 cm Wratten neutral density filters (Wratten 96; Eastman Kodak; Rochester, NY) with variable optical densities (ODs). Different optical transmissions were obtained by putting more than one filter over the other one (Table 1). The distance between the photic stimulator and the eye of rabbit was kept in 15 cm and the illumination at the level of rabbit eye was measured by a luminometer (TES digital luminometer; TES Electronic Corp.; Taipei, Taiwan) in every photic stimulations. The trigger input port of data acquisition device (DAQ) (PowerLab 26T; ADInstruments Pty Ltd; New South Wales, Australia) was connected to output port of an unemployed EEG device (Nihon Kohden; EEG 4318) by BNC cable (Fig. 1). The PowerLab traces were automatically synchronized with the flash by the synchronizing trigger output of the flash unit. In order to see a marker on ERG trace for every light stimulus, we have adjusted the trigger marker settings as ‘on’ in Scopus software (ADInstruments Pty Ltd; New South Wales, Australia). The signals were filtered with a pass-band of 1–300 Hz. The trace began 10 s prior to the flash in order to provide a pre-flash baseline of the bioelectric signal recorded from the eye. In order to keep the inter-stimulus interval at least 5 seconds for getting stronger responses, we have used 0.1Hz temporal frequency in photic stimulation for scotopic ERG. Low (1 Hz) and high (10 Hz and 30 Hz) temporal frequencies have been used in light stimulation to test cone system function (for photopic ERG). All frequencies were manually adjusted and managed by the control button over the EEG device (Fig. 2).

### Table 1. Optical densities and transmissions of neutral density filters used in the study and stimulus strength obtained by the filters are shown.

<table>
<thead>
<tr>
<th>ERG Condition</th>
<th>Optical Density</th>
<th>Transmission (%)</th>
<th>Stimulus Strength (log cd.s/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photopic</td>
<td>0.3</td>
<td>50</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>25</td>
<td>1.69</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>1.30</td>
</tr>
<tr>
<td>Scotopic</td>
<td>0.3</td>
<td>50</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>25</td>
<td>1.69</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>63</td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>2.30</td>
</tr>
</tbody>
</table>

In order to see a marker on ERG trace for every light stimulus, we have adjusted the trigger marker settings as ‘on’ in Scopus software (ADInstruments Pty Ltd; New South Wales, Australia). The signals were filtered with a pass-band of 1–300 Hz. The trace began 10 s prior to the flash in order to provide a pre-flash baseline of the bioelectric signal recorded from the eye. In order to keep the inter-stimulus interval at least 5 seconds for getting stronger responses, we have used 0.1Hz temporal frequency in photic stimulation for scotopic ERG. Low (1 Hz) and high (10 Hz and 30 Hz) temporal frequencies have been used in light stimulation to test cone system function (for photopic ERG). All frequencies were manually adjusted and managed by the control button over the EEG device (Fig. 2).
Interpretation of ERG
LabChart Reader software (LabChart Reader v8.0; ADInstruments Pty Ltd; New South Wales, Australia) was used for ERG analysis. Scotopic (dark-adapted) and photopic (light-adapted) amplitudes of the a-wave and b-wave were measured. A-wave amplitudes were measured from the preresponse baseline to the trough of the negative wave and reflect photoreceptor function (outer retina). B-wave amplitudes were measured from the trough of the a-wave to the peak of the b-wave and reflect Muller cell and bipolar cell function (inner retina). For oscillatory potentials of the 10- and 30-Hz flicker responses, the amplitude (OPs) was measured from the preresponse baseline to the peak. Implicit time for a- or b-wave was obtained by calculating the time between the stimulus and the top of the a- or b-peak. During analysis, we have used the average of the 10 ERG responses for every light stimulus with different strengths. Mean amplitudes of a- and b-waves were obtained by calculating the average of all a- and b-waves in all stimulus strengths.

Intravitreal saline injection
The needle was inserted infero-temporally through the conjunctiva into the pars plana 3–5 mm from the limbus. The globe was oriented so that the needle track was axial with no rotational movement. The needle was directed posteriorly towards the optic nerve to the central vitreous approximately 5 mm posterior to the apex of the posterior capsular pole. The needle was observed through the pupil, and care was taken not to nick the lens capsule or disrupt the vitreous. 0.05 ml isotonic saline were injected into the central of vitreus of the rabbit’s right eyes after anesthesia. A cotton-tipped applicator was placed over the injection site for 30 s to minimize risk of reflux.

3. Results
We have obtained all components of an ERG measurement in this experimental set; such as, a- and b-waves, oscillatory potentials (OPs), photopic-negative response (PhNR), and i-wave (Fig. 3). ERG responses were obtained by short-duration light stimuli with different strengths in scotopic (-2.69; -1.69; 0.00; 0.30; 0.69; 0.90; 1.10; 1.30; 1.69; 2.00 log stimulus energy (log cd.s/m²)) and in photopic conditions (1.3; 1.69; 2.0; 2.10; 2.30 log stimulus energy (log cd.s/m²)). ERG traces in scotopic and photopic conditions, and flickers are shown in Fig. 4.

Before saline injection, mean amplitude of a- and b-waves were -38.5 µv and 116.5 µv; respectively. Mean amplitude of a- and b-waves after saline injection were -35.3 µv and 104.4 µv, respectively. Implicit time of a- and b-waves before injection were
0.019 s and 0.045 s, and they were 0.015 s and 0.041 s after injection, respectively (Fig. 5). There was no statistically significant difference between before and after saline injection for these parameters (p>0.05).

![Fig. 5](image)

Fig. 5. Mean amplitudes of waves, the stimulus time and mean implicit time of waves are demonstrated in the ERG traces for baseline (A) and after saline injection (B).

4. Discussion

Experimental set of ERG may not be always available for all researchers. In our study, we defined a cost-effective and easily available technique to perform experimental ERG for the studies of retinal toxicity.

We have used an unemployed EEG device for photic stimulation. It can be easily synchronized with data acquisition device and we obtained precise measurements of implicit time in ERG traces by this synchronization. As a data acquisition device, we have used PowerLab 26T in our study. It has integrated data recording featuring a dual Bio Amp, an isolated stimulator and trigger input, and it includes Scope and LabChart software to quantify the ERG traces and analysis the data. This kind of devices are usually used in nearly all physiology laboratories for multiple purposes. By the control panel of EEG device (Fig. 2, it can be easy to change the frequency of the light stimulus. We have used 0.1Hz temporal frequency in photic stimulation for scotopic ERG and have used an average of 10-12 repeated flashes at 1Hz temporal frequency for photopic ERG to get maximum cone response. This represents the less variable intra-individual factor and thus enable comparison between the same animal from a session to another one (Rosolen et al., 2008; Perlman, 2009). So we have obtained similar results in ERG measurements before and after the saline injection for the amplitudes and implicit time of a- and b- waves. As a corneal electrode, we used ERG-jet equipped with a golden-metallic ring that does not generate artificial pupil and it is generally used for species with larger corneal diameter; such as human and rabbits. There is commercially available electrodes for an alternative electrodes to these expensive contact lens electrode, the fiber electrodes such as DTL are more popular (Dawson et al., 1979). DTL electrodes offer several advantages: painless even after several hours, no need for topical anesthesia and disposable. However, they must be used with great care and their positioning controlled as even minor changes in position could have a significant impact on the amplitude of the ERG parameters.

Ganzfeld dome provides standardized media for homogenous light stimulus and it allows researcher to take ERG response from both eyes of the rabbit at the same time. Although we did not use any Ganzfeld dome in our study, we obtained similar results in ERG response before and after saline injection. Partial sphere with nearly 0.40 m diameter would be used for this purpose. Internal surface of it should be painted with a high reflectance, spectrally flat coating (for example: Avian D high reflectance coating, Avian Technologies, Wilmington, OH) (Harrison et al., 2005).

In the study, we have obtained all components of an ERG measurements such as, a- and b- waves, OPs, PhNR, and i-wave. The changes in these components by altering OPs the strength of light stimulus was clearly seen in Fig. 4. It is now accepted that in photopic conditions the leading edge of the a-wave (first 10 ms) reflects the photoreceptor response to bright light (Hood and Birch, 1996). It should be noted that there is no pure rod ERG a-wave as such because in scotopic condition, an a-wave is recordable only in response to flashes of light intensities, which are in the photopic range. These responses are usually referred to as mixed cone and rod ERG because both photoreceptors are claimed to contribute to their genesis (Rosolen et al., 2005). In the study, a-wave has appeared first at the level of 0.30 log stimulus energy (log cd.s/m²) in scotopic ERG trace, and it was shown that a-wave has been augmented by the increase in stimulus strength for both scotopic and photopic conditions and has achieved the maximum at the level of 2.10 log stimulus energy (log cd.s/m²) in our study. The b-wave, that was attributed to post-receptoral elements (ON-bipolar cells), has appeared first at the level of -1.69 log stimulus energy (log cd.s/m²) in the study, and it was also correlated with the stimulus strength. We obtained decay in implicit time after saline injection. Even if there was a difference in implicit time (0.004 s) before and after saline injection, it should be noted that faster than normal responses are usually considered as a variation of normal (Rosolen et al., 2008).

The OPs of ERG, that are best visible with bright light stimulation, can be used to assess the functional integrity of the inner retina (Heynen et al., 1985; Wachtmeister, 1987). The PhNR is a negative-going wave that occurs following the b-wave and it was seen that PhNR have been more visible after the stimulus level of 0.30 log stimulus energy (log cd.s/m²) in ERG trace. Since the origin remains undetermined, the PhNR is significantly reduced in human patients with primary open angle glaucoma (Viswanathan et al., 2001), anterior ischemic optic neuropathy (Rangaswamy et
al., 2004) and other optic nerve neuropathies (Gotoh et al., 2004), consistent with an origin in ganglion cells or their axons. The i-wave is a positive deflection that occurs approximately 20 ms after the b-wave which can easily be identified in a number of animal species (Rosolen et al., 2004). It is thought that i-wave might be attributed to the activation of the retinal ganglion cells and the optic nerve including the chiasm (Rousseau et al., 1996). The amplitudes of PhNR and i-wave were related with the strength of the light stimulus in our study.

We have obtained all components of an ERG measurement in our study, and we have used an unemployed EEG device for photic stimulation. It can be easily synchronized with data acquisition device and we obtained precise measurements of implicit time in ERG traces by this synchronization. According to our definition of the experimental set, researchers may easily perform experimental ERG, and precise and more accurate ERG measurements can be obtained for the studies of retinal toxicity.

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REFERENCES


