·Basic Research·

Safety of intravitreal triamcinolone acetonide: an electrophysiologic and histopathological study in rabbits

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Abstract

• AIM: To evaluate the retinal safety of various doses of intravitreal triamcinolone acetonide (TA) in rabbits.

• METHODS: Thirty New Zealand albino rabbits were divided into five groups (six animals each). In group 1 (control group), each animal received a single intravitreal injection of 0.1mL phosphate buffered saline. In groups 2, 3, 4 and 5, each rabbit received a single intravitreal injection of 4, 8, 16 and 32mg of TA, respectively. Each dose was contained in 0.1mL phosphate buffered saline. Clinical ocular examinations were performed before the injection and on the 1st, 3rd, 10th and 17th post-injection days. A standard dark adapted electroretinogram (ERG) was obtained before injection and on the 3rd, 10th and 17th post-injection days. After 17d, animals were sacrificed and their eyes prepared for pathological examination.

• RESULTS: By monitoring ERG as a functional index for the retina, intravitreal injection of 4mg TA showed no significant ERG changes. At doses of 8, 16 and 32, hyper-abnormal responses in a- and b- waves of ERG were detected on the 3rd post-injection day. These changes gradually returned back to normal limits after 17d. Histopathological examination of the retina of all animals showed no pathological changes.

• CONCLUSION: High doses of intravitreal TA seemed to have enhancing effects on the retinal function with gradual return to normal limits with no pathological changes detected in examined eyes.

• **KEYWORDS:** triamcinolone acetonide; intravitreal injection; electroretinogram

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INTRODUCTION

Triamcinolone acetonide (TA), a synthetic glucocorticoid, is the most commonly used glucocorticoid for intravitreal injection because its low intraocular solubility allows for long duration sustained effect. Intravitreal TA (IVTA) has been used for treatment of intraocular diseases, such as long standing macular edema due to retinal vein occlusion or branch retinal vein occlusion, diffuse diabetic macular edema, neovascular glaucoma, ocular hypotony, chronic uveitis and age related macular degeneration[1-5].

Triamcinolone acetenoid suppresses inflammation *via* several mechanisms. It demonstrates potent inhibitory effects on mitogen-activated protein kinase (MAPK) signaling pathways through the induction of MAPK-1 and this inhibits the expression of multiple inflammatory genes[6]. In addition, TA inhibits cyclooxygenase, interleukin-6 (IL-6) and reduces vascular permeability[7]. Moreover, it increases the resorption of fluid through the retinal pigment epithelium (RPE) and down regulates the production of vascular endothelial growth factor (VEGF-A)[8].

Because pevious studies suggested that the effect of intravitreal triamcinolone acetonide showed a dosage dependency, many researchers attempted the use of high doses of IVTA, aiming to reduce the frequency of required intravitreal re-injections [9,10]. Different doses of intravitreal TA, varying from about 4mg to 30mg, has been employed with contradictory results as regard efficacy, toxicity and duration of effect[10]. In rabbit's eyes, after a single intravitreal injection of TA, no influence on global ERG responses was detected [11]. On the other hand, in other studies, TA induced clear toxic effects on RPE cells, retinal Müller glial cells and retinal neurosensory cells [12-14]. Because IVTA is widely and increasingly used in various ocular diseases, more efficient and specifically targeted effects of corticosteroids are needed. To achieve these aims, ensuring safety of the intravitreal

triamcinolone influence on eye tissues is essential. The present study was undertaken to investigate the effect of various doses of IVTA on the function (assessed by ERG) and the structure (assessed by histopathology) of the retina in rabbit eyes.

MATERIALS AND METHODS

Materials Thirty New Zealand albino rabbits of both sexes, weighing between 2-2.5kg, aged between 7 to 8 months, were used in this study. We tried to have a nearly similar age in the studied groups to have same degree of retinal maturation. Animals were used in accordance to the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research. The experiment was approved by our institutional ethical committee. All through the experiment duration, rabbits were housed in separate cages, fed standard laboratory food and allowed free access to water in room temperature with 12h light-dark cycle in the animal house of the Research Institute of Ophthalmology.

Animals were divided into five groups randomly using random number generator; each comprised of six rabbits. In group 1 (control group), six rabbits were subjected to a single intravitreal injection of 0.1mL phosphate buffered saline. Rabbits of the remaining four groups (groups 2, 3, 4 and 5) were injected once with triamcinolone acetonide (Sigma-Aldrich, Germany) by intravitreal injection at doses of 4, 8, 16 and 32mg respectively. Each dose was suspended in 0.1mL phosphate buffered saline. In studied animals, only right eyes were injected intravitreally with total of six eyes in each group.

Methods

Intravitreal injection Animals were anesthetized before the intravitreal injections by intramuscular injection of 50mg/kg ketamine hydrochloride (Ketamar, Amoun, Egypt) and 5mg/kg lignocaine hydrochloride (Xylocaine, Astra-Zeneca, Sweden). Pupils were dilated by topical instillation of 2.5% phenylephrine hydrochloride (Phenylephrine, Misr, Egypt) and 1% tropicamide (Mydriacyl, Alcon, Belgium).

After topical instillation of 0.4% benoxinate hydrochloride (Benox, Eipico, Egypt), eyes were washed with several drops of 5% povidone iodide. Anterior chamber paracentesis using a 27-gauge needle was performed before intravitreal injection to avoid high post-injection intraocular pressure and to minimize drug reflux after injection. The intravitreal injection was performed using a 27-gauge needle through a site 2-mm posterior to the superior-temporal limbus and the needle tip was directed to the mid-vitreous under direct visualization with external illumination of a surgical microscope. The needle was held in place for a few seconds before withdrawal to prevent reflux from the entry site. The central retinal artery was observed with indirect ophthalmoscopy to ensure its patency after each injection. After intravitreal injection, eye drops containing an antibiotic-corticosteroid combination (Dexatrol, Eipico, Egypt) was applied to eyes three times daily for three days.

Ophthalmologic examination Ophthalmologic clinical examinations were performed immediately before injections (baseline) and on 1st , 3rd, 10th and 17th post injection days. Examinations included slit lamp anterior segment examination, and detailed funduscopic examination of studied eyes. A baseline standard ERG was obtained one day before the intravitreal injection and on 3rd, 10th and 17th postinjection days.

Electrophysiological tests Electroretinogram (ERG) was performed using the Reti-com system (Roland-Consult). After anesthesia (as described above before intravitreal injection) rabbits were dark adapted for at least 30min after pupil dilation. The active electrode was placed near the margin of the lower eyelid, the reference electrode was placed on the forehead and the earth electrode was clipped to the earlobe. Recording of the combined response was carried out using a mini-Ganzfeld flash stimulus with a maximum intensity of 3.0cd-s/m^2s with no background intensity. ERG signals were amplified and filtered (0.3-300Hz). Amplitude was measured from the 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. Latency was measured from the beginning of the stimulus to the negative peak of the a-wave (a latency), and to the following positive peak of the b-wave (b latency). Data were expressed as mean±SD. Analysis of variance (ANOVA) with post-Hoc multiple comparisons were performed to compare responses between and within groups. P value was considered significant if $P<0.05$.

Histopathological examination Animals were euthanized on 17th day post injection with overdose of intracardiac ketamine and xylazine. Globes were enucleated, and fixed immediately in 10% buffered formalin. Eyes were sectioned horizontally to obtain a pupil-optic nerve section and examined macroscopically. Tissues were then processed and embedded in paraffin, sectioned at a thickness of 5μm, and stained with hematoxylin and eosin. Light microscopy was used for histological examination.

RESULTS

Ophthalmologic Examination Baseline slit lamp examination and on the 1st, 3rd, 10th and 17th post injection days, did not reveal cell flare or hypopyon in the anterior chamber of treated eyes or control eyes. Faint posterior subcapsular cataract was observed in one eye in group 4, this could be attributed to touch of the lens during injection. Fundus examination showed normal retinal appearance with no retinal detachment. Clumped white triamcinolone precipitates were seen within the vitreous ophthalmoscopically in groups 2, 3, 4 and 5. It seemed that increasing the dose caused the white precipitate to be more dense. Triamcinolone

Table 1 Mean values of a- and b-wave amplitudes (in microvolt) and a- and b-wave latencies (in seconds) on days 3, 10 and 17 after a single intravitreal injection of 0.1mL phosphate buffered saline in control group 1 and injection with 4, 8, 16 and 32 mg TA/0.1mL phosphate buffered saline in groups 2, 3, 4 and 5 respectively

Groups		a-wave amplitude (μV)	a-wave latency (s)	b-wave amplitude (μV)	b-wave latency (s)
1	3rd day	5.64±1.27	15.17±0.76	28.43±1.88	35.23±1.52
	10th day	5.63±1.28	13.52±4.16	28.30±1.82	34.85±1.22
	17th day	5.53±1.49	14.70±0.92	29.22±2.5	35.34±2.0
2	3rd day	5.54±1.14	15.13±0.74	27.38±1.74	34.92±0.96
	10th day	5.21±0.87	15.10±0.81	28.37±1.6	35.03±1.17
	17th day	5.04±0.99	15.27±1.01	29.25±1.26	35.01±0.90
3	3rd day	[1]9.88±1.06	15.4±0.67	[1]53.82±2.56	38.25±1.79
	10th day	4.45±0.27	12.95±1.29	29.63±1.37	36.17±1.23
	17th day	6.97±0.40	14.93±0.28	27.05±0.31	33.93±0.62
4	3rd day	[1]9.83±0.99	15.68±1.17	[1]54.37±2.59	38.03±1.94
	10th day	[1]11.0±0.21	16.02±0.35	[1]55.03±0.28	37.73±0.30
	17th day	6.87±0.51	14.63±0.42	35.30±1.04	35.82±0.79
5	3rd day	[1]8.87±0.27	[1]17.25±0.39	[1]55.70±1.50	[1]40.47±0.70
	10th day	[1]11.17±0.33	15.18±0.54	[1]55.08±0.60	38.01±0.35
	17th day	5.03±0.28	13.08±0.25	31.38±0.49	34.02±0.70

Data is expressed as mean±SD, n=6, μV: Microvolt; TA: Triamcinolone acetonide. [1]significant difference (P<0.05) as compared to group 1.

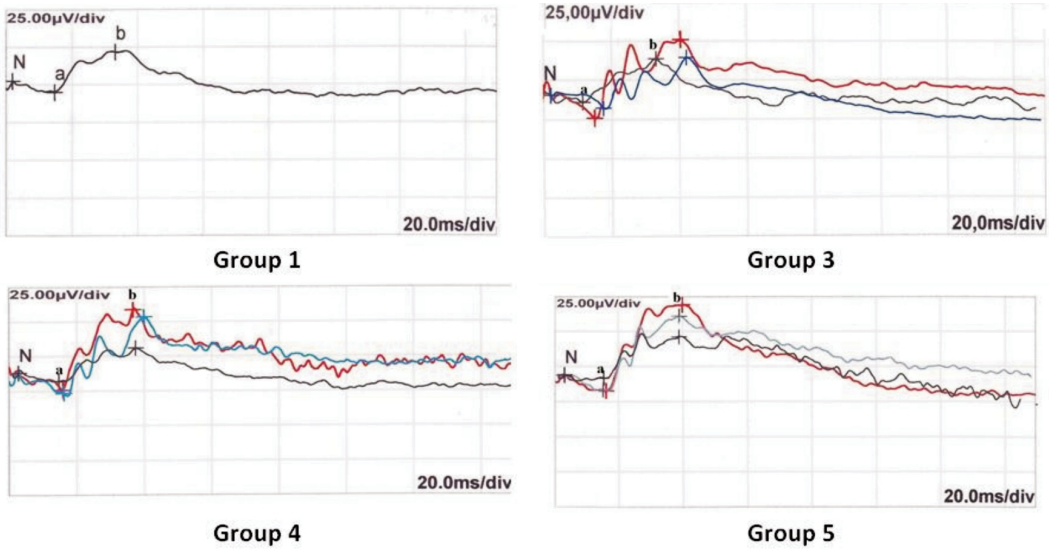

Figure 1 Showed the electroretinographic (ERG) recordings of selected rabbit eyes in groups 1, 3, 4 and 5 respectively On the 3rd post-injection day (red lines), the ERG combined response demonstrated hyper-abnormal a- and b-wave amplitudes in groups 3, 4 and 5. On the 10th post-injection day (blue lines), the hyper-abnormal responses were demonstrated in groups 4 and 5, while in group 3 amplitudes returned to normal values. On the 17th post-injection day (black lines), ERG responses returned to normal values in all injected eyes.

gradually disappeared in group 2 by the 17th day. Meanwhile, groups 3, 4 and 5 showed remnants of triamcinolone powder on the 17th day.

Electrophysiological Tests Statistical analysis of the pre-injection ERG showed no significant differences within and between the study groups (P>0.05). Post-injection ERG recordings are represented in Table 1 and Figure 1. Table 1 shows the mean value of a- and b-wave amplitude and latency in studied groups on the 3rd, 10th and 17th day after injection. In control group 1, a- and b-wave amplitudes and latencies were normal on the 3rd, 10th and 17th day with no significant difference between these values and pre-injection values.

ERG recordings in group 2 (4mg triamcinolone) showed no significant changes as compared to control (group 1) on all days of examination. On the 3rd post-injection day, animals which were injected with triamcinolone in doses of 8mg (group 3), 16mg (group 4) and 32mg (group 5) showed markedly increased a- and b-wave amplitudes (hyper-abnormal responses) that were significantly high (P< 0.001) as compared to control group 1.

In group 3, a- and b-wave amplitudes returned to normal values with no significant difference from control group 1 on the 10th and 17th post-injection day. However, hyper-abnormal responses were still observed in group 4 and group 5 on the 10th post-injection day and a- and b-wave amplitudes were significantly increased as compared to control group 1 (P<0.001). On the 17th day, a- and b-wave amplitudes of group 4 and group 5 returned to baseline values as no significant difference was observed between control group 1 and other treated groups.

As regards a- and b-wave latencies, there were no significant differences between treated groups and the control (group1). The only exception was observed in group 5 which showed

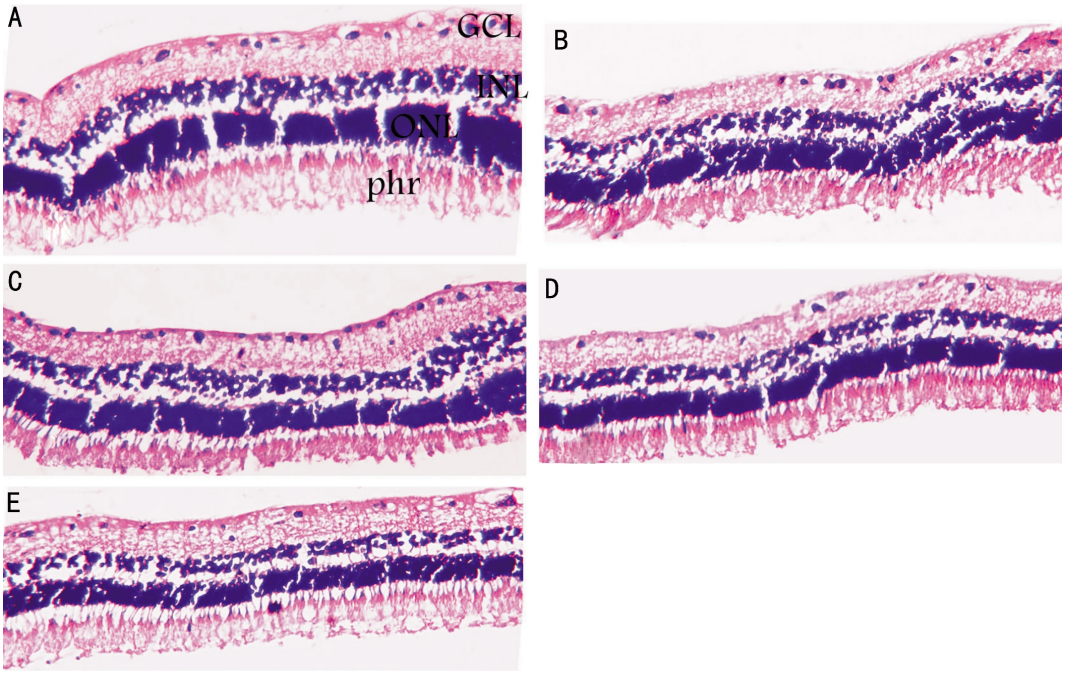

Figure 2 Light photomicrograph of selected retinal sections A: A control eye (injected intravitreally with 0.1mL phosphate buffered saline. It shows normal appearance of retinal layers, photoreceptors (Phr.), outer nuclear layer (ONL), ganglion cell layer (GCL); B, C, D, E: retinas of groups 2, 3, 4 and 5 injected intravitreally with triamcinolone acetonide at doses of 4, 8, 16 and 32mg/0.1mL phosphate buffered saline, respectively. There were no distiguishible changes between studied groups (Hematoxylin and eosin, ×200).

significant delay in a- and b-wave latencies (P=0.002 and 0.001, respectively) on the 3rd post-injection day, as compared to the control group. However, they returned to normal values on the 10th and 17th post injection day with no significant difference from the control group 1.

Figure 1 shows ERG recordings of selected eyes from groups 1, 3, 4 and 5 (group 2 was not included as the recordings were similar to the control group).

Histopathological Examination Hematoxylin and eosin-stained sections disclosed normal light microscopic appearance of the retina, retinal pigment epithelium and choriocapillaris in all studied animals.

Figure 2 shows photomicrographs of some retinas of examined groups 1, 2, 3, 4 and 5.

DISCUSSION

Because TA suspension provides longer-lasting anti-inflammatory, antiproliferative, antiangiogenesis and antipermeability effects compared with other steroid preparations, it has become more widely used in treating wide variety of vitreoretinal diseases [15]. In the present study, we investigated the ERG and histopathological effect of increasing doses of TA after single intravitreal injection in albino rabbits. Many risks of intravitreal TA injection were reported which may be procedure-related (such as vitreous hemorrhage, bacterial endophthalmitis, pseudo-endophthalmitis and retinal detachment), corticosteroid-related (such as cataract and elevated intraocular pressure) or preservative toxicity-related of commercial preparations [16,17]. The preservative benzyl alcohol and suspending agents sodium carboxymethylcellulose and polysorbate 80 were reported to produce loss of photoreceptor outer segments, RPE proliferation and localized vitritis [18,19]. Except for faint posterior subcapsular cataract observed in one eye, such complications were not detected in the present study. It can be explained by strictly aseptic precautions during injection and use of preservative free TA. Also, the small sample size and short duration of the experiment may played a role. The low rate of complications in the present work was in agreement with McGee *et al* [20], who mentioned no clinical complications after single intravitreal injections of 4, 16 and 25mg TA in rabbits.

ERG is a useful examination to study retinal function. The ERG a-wave is obtained primarily from the maximal combined response and it reflects the photoreceptor function. Physiologically, a-wave arises from the light evoked closure of sodium channels along the outer segment plasma membrane of receptor cells. The b-wave results from the current flow along Müller cells in response to increased extracellular potassium ion concentration. It is highly dependent on bipolar cells within the inner nuclear layer and hence on the retinal circulation[21].

In the present study, intravitreal TA in doses of 8, 16 and 32mg showed transient hyper-abnormal responses on the 3rd post-injection day with gradual return to normal responses thereafter. These hyper-abnormal responses were detected with many conditions such as albinism, atypical cone dystrophies, optic nerve sectioning, optic neuropathies, vascular occlusions, ischemia and uveitis [22]. Also, it was

reported with some drugs including corticosteroids, low-dose barbiturates and carbon disulphide poisoning. It was suggested that such responses can be due to irritation of the retina or suppression of inhibitory pathways in outer and middle retinal layers [22]. In accordance with the results of the present study, Dierks *et al* [11], suggested that TA therapy might augment rod-driven electroretinographic responses. In addition, it was mentioned that administration of TA could reduce the production of VEGF-A, arachidonic acid and prostaglandins allowing reactivation of fluid clearance by Müller cells. These processes could lead to decrease of Müller cell proteins, reduction of the osmotic swelling of Müller cells and efflux of potassium ions which could partially explain the hyper-abnormal responses encountered with TA [23]. In the present investigation, ERG changes were transient and returned to normal by the 17th post-injection day and this correlated well with histopathological results which showed no retinal toxicity manifestation.

Results of this study were also in agreement with results of Ruiz-Moreno *et al* [10], who mentioned that intravitreal TA in doses of 4, 20 and 30mg did not seem to have acute toxic effects on the retinal function and structure in albino rabbits. Moreover, single intravitreal injections of 4, 16 and 25mg TA resulted in normal histological and ERG retinal findings[20].

McGee *et al* [20], observed basophilic material, which was presumed to be drug, in the vitreous with clumps adjacent to the retinal surface which was similarly detected in our study. This precipitation of triamcinolone, which is a lipophilic slow release large particles, could be explained by the early sacrifice of animals before the crystals had cleared, as triamcinolone was mentioned to remain in the vitreous up to six months after injection [24]. It is worth to mention that triamcinolone crystals in direct contact with retinal cells in cell culture have been observed to cause cell damage, perhaps due to the lack of the potentially protective effect of the ILM or vitreous[25].

In the present work we prepared TA; firstly to ensure consistent dosing and secondly to avoid the toxic effect of the solvent agent benzyl alcohol in commercially available solutions, since several reports have recommended removal of the solvent agent [18]. It was mentioned that commercial triamcinolone applied to cultured primary rat retinal cells induced retinal oxidative injury suggesting toxic potential[26]. Additionally, it was reported that cases injected with preservative free TA had lower rate of noninfectious endophthalmitis[13].

It was mentioned that commercial preservative-containing TA injected intravitreal in doses of 4, 8, and 20mg induced prominent retinal damage manifested by damage to the photoreceptor outer segments and RPE [12]. However, when preservative-free TA was injected subretinally , Maia *et al*[13], disclosed disturbance in photoreceptor segments This showed that the direct toxic effect of the drug was observed when it was injected subretinally, with no protective effect of internal limiting membrane or vitreous It was suggested that TA in higher doses than 4mg, *in vitro* studies, could affect the DNA rich mitochondria in the inner segment of photoreceptors and induce a non-apoptotic cell death of the RPE cells and the Muller cells [27]. Thus, keeping the preservative-free TA injection in the mid vitreous, as we did in this study, might add to the safety of the procedure. Some limitations of the present study must be considered. Firstly, TA was injected into a smaller vitreous volume, less than 2mL in the rabbit, as opposed to 4-5mL in humans which would lead to less drug concentration if the same dose of TA used in rabbits was injected in humans. Therefore, extrapolation from animal to human studies should be done with caution. Additionally, histological examination was confined to pupil-optic nerve sections. Consequently, we could not comment on the presence or absence of localized photoreceptor damage outside these sections. Thus, further histopathological testing is required to evaluate retinal layers particularly the photoreceptor layer. Another factor was the topical antibiotic/corticosteroid drops applied drops applied post-injection could have a confounding result because topical eye drops may reach the retina.

In conclusion, based on electrophysiology and histopathology, the present study showed no evidence of retinal toxicity resulting from high doses of intravitreal TA in rabbits. However, further investigations are needed in animal eyes to examine all retinal layers microscopically and in human eyes to determine the safe intravitreal TA dose.

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