

## SCIENTIFIC REPORT

## Effect of triamcinolone acetonide on proliferation of retinal endothelial cells in vitro and in vivo

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**Aim:** To assess the effect of crystalline triamcinolone acetonide on retinal endothelial cell proliferation in vivo and in vitro.

**Methods:** For in vitro analysis, a sprouting assay was employed. Bovine retinal endothelial cells were stimulated with basic fibroblast growth factor (bFGF) and incubated with different concentrations of triamcinolone acetonide (0.05 mg/ml to 8 mg/ml). For in vivo analysis, a retinopathy of prematurity (ROP) model was used. 16 C57BL/16 mice were exposed to 75% oxygen from postnatal day 7 to day 12. On day 12, triamcinolone acetonide was intravitreally injected into one eye ("study eye") and isotonic saline into the contralateral eye ("control eye"). On day 17, the mice were sacrificed and the eyes removed for quantitative analysis of preretinal neovascularisation. Four non-exposed mice served as negative control.

**Results:** The sprouting assay demonstrated a dose dependent inhibition of bovine retinal endothelial cell proliferation from 0.05 mg triamcinolone acetonide/ml (no inhibition) to 3 mg triamcinolone acetonide/ml (complete inhibition). Dosages of more than 2 mg/ml resulted in cytotoxic changes of endothelial cells. The ROP model demonstrated a significantly lower neovascular cell count of 58% in the study group compared to the control group (6.35 (SD 2.1) cells per histological section versus 14.9 (SD 5.3) cells;  $p < 0.005$ ).

**Conclusions:** Triamcinolone acetonide inhibits bFGF induced proliferation of retinal endothelial cells in vivo and in vitro. These findings contribute to understanding the mode of action and effects of triamcinolone acetonide on retinal neovascularisation.

Neovascular diseases of the eye such as proliferative diabetic retinopathy and retinopathy of prematurity (ROP), often result in irreversible visual acuity loss or even blindness.<sup>1,2</sup> They are usually treated by panretinal argon laser coagulation, destroying ischaemic retinal tissue, the production site of angiogenic factors.<sup>3</sup>

Recent research has focused on medical, non-invasive inhibition of ocular angiogenesis. An increasing number of these studies have indicated that intravitreal injection of triamcinolone acetonide has an anti-oedematous, anti-angiogenic effect in neovascular ocular diseases such as exudative age related macular degeneration (ARMD), macular oedema secondary to diabetes, and central retinal vein occlusion.<sup>4–15</sup> Correspondingly, several experimental studies using animal models indicate an angiostatic effect of triamcinolone acetonide on retinal neovascularisation in vivo.<sup>16–20</sup>

In vitro studies, however, examining the effect of triamcinolone acetonide on retinal endothelial cells have

not been performed until now. Therefore, the mode of action of triamcinolone acetonide on retinal endothelial cells is unclear. It is furthermore unknown, whether triamcinolone acetonide is capable of suppressing proliferating retinal endothelial cells directly. To answer this question, a sprouting assay as in vitro model was employed. In order to demonstrate an in vivo effect of triamcinolone acetonide on retinal neovascularisation, an established ROP model was employed.<sup>21,22</sup>

## MATERIALS AND METHODS

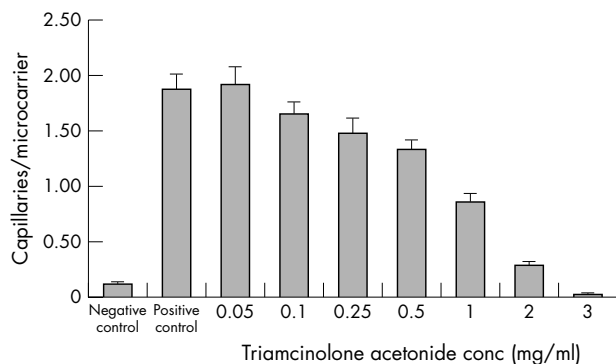
## Sprouting assay

Bovine retinal endothelial cells were cultivated onto Cytodex 3 microcarrier (Amersham Biosciences Europe GmbH, Freiburg, Germany) for 48 hours. For gel preparation, bovine fibrinogen (Calbiochem-Novabiochem GmbH, Schwalbach, Germany) was dissolved in phosphate buffered saline (PBS) pH 7.4 (concentration 5 mg/ml) and finally adjusted to an end concentration of 1.8 mg/ml with Dulbecco's PBS pH 7.4 (Invitrogen GmbH, Karlsruhe, Germany). Aprotinin (Trasylol) 200 U/ml was used as a protease inhibitor. The fibrinogen was placed into 12 well plates (Corning GmbH, Wiesbaden, Germany). Confluent microcarriers were seeded into each well. The fibrinogen was polymerised with thrombin (Sigma-Aldrich Chemie GmbH, München, Germany). Basic fibroblast growth factor (bFGF, Invitrogen GmbH, Karlsruhe, Germany) in a concentration of 2 ng/ml and crystalline triamcinolone acetonide in concentrations ranging from 0.05 mg/ml to 8 mg/ml were added to each well. Other mediators such as ECGS (endothelial growth supplement, Promocell GmbH, Heidelberg, Germany) were tested.

The length of the experiments varied from 48–60 hours depending on the positive control (bFGF 2 ng/ml, approximately 1–2 capillaries per microcarrier). As negative control, bovine retinal endothelial cells were incubated with isotonic saline. Experiments were performed in endothelial cell basal medium (PromoCell) containing 0.5% fetal calf serum (FCS).

For statistical analysis, tube formation assays were carried out in duplicate and the experiments were re-performed on three different occasions. Using a light microscope (100× magnification), all capillaries that were at least as long as the bead diameter (175 µm) were counted. Results are shown as mean count of capillaries (SD). Mean values were compared using the Mann-Whitney U test and a  $p$  value  $< 0.05$  was considered statistically significant.

**Abbreviations:** ARMD, age related macular degeneration; bFGF, basic fibroblast growth factor; BRECs, bovine retinal endothelial cells; ECGS, endothelial cell growth supplement; FCS, fetal calf serum; MC, microcarrier; PBS, phosphate buffered saline; ROP, retinopathy of prematurity; TA, triamcinolone acetonide; VEGF, vascular endothelial growth factor



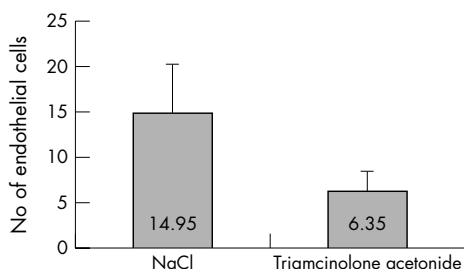
**Figure 1** Histogram of the sprouting assay showing the number of capillaries per microcarrier (mean (SD)) after incubation with basic fibroblast growth factor and stimulation with increasing concentrations of triamcinolone acetonide (TA) (0.05–8 mg/ml). Bovine retinal endothelial cells (BRECs) incubated with dosages of TA higher than 2 mg/ml showed total inhibition. As negative control, isotonic saline was used. For positive control, BRECs were treated with basic fibroblast growth factor only (bFGF 2 ng/ml).

### Retinopathy of prematurity model

The retinopathy of prematurity model was used as published.<sup>21–22</sup> Briefly, newborn C57BL/6 mice were exposed to 75% oxygen from postnatal day 7 to postnatal day 12 with their nursing mothers. On the 12th day the mice were returned to room air. Crystalline triamcinolone acetonide (Volon A; Bristol-Myers-Squibb) was prepared by centrifuging an entire ampule containing 40 mg triamcinolone acetonide in 1 ml benzene alcohol, removing the supernatant with the vehicle substance, and replacing it with Ringer's solution. This procedure was repeated twice. Diethylether was used to anaesthetise the mice for intravitreal injections. Using a Hamilton syringe, 1 µl of triamcinolone acetonide (40 mg/ml) was injected into the vitreous cavity of the right eye at the corneoscleral junction at the 6 o'clock position. The contralateral eye received an intravitreal injection of 1 µl isotonic saline in the same position. Four normal mice without exposure to oxygen served as negative controls.

On postnatal day 17 the mice were sacrificed and the eyes enucleated and fixed in 4% formalin. For quantitative assessment of retinal neovascularisation, Periodic acid Schiff (PAS) stained serial sections were used according to the published method. Neovascularisations were quantified by counting the vascular cell nuclei on the vitreal side of the inner limiting membrane of the retina.

For statistical analysis, the results are shown as mean (SD) count of neovascular nuclei. Mean values were compared



**Figure 2** Histogram showing the count of neovascular cells (mean (SD)) on the vitreal surface of the inner limiting membrane of the retina in 16 mice with an experimental model of retinopathy of prematurity and receiving an unilateral intravitreal injection of 1 µl (40 mg/ml) of triamcinolone acetonide (triamcinolone group), and an intravitreal injection of saline into the contralateral eye (NaCl group).

using the Mann-Whitney U test, and a value less than 0.05 was considered statistically significant.

All experiments were performed in accordance with the guidelines for the design and conduct of animal experiments of the Association for Research in Vision and Ophthalmology and were approved by local animal care authorities.

### RESULTS

The bovine retinal endothelial cells (BRECs) were stimulated with bFGF and then incubated with triamcinolone acetonide in a range from 0.05 mg/ml to 8 mg/ml. The proliferation response of the negative control was minimal (fig 1), in contrast with the positive control (bFGF 2 ng/ml) which demonstrated a maximal response. The sprouting assay revealed a dose dependent inhibition of BRECs by triamcinolone acetonide (fig 1): No inhibition was recorded after incubation with triamcinolone acetonide 0.05 mg/ml, whereas BRECs incubated with a dosage of triamcinolone acetonide of more than 2 mg/ml showed complete inhibition. With increasing dosages (higher than 2 mg/ml), the BRECs showed progressive cytotoxic changes. Taking all samples, the proliferation of bovine retinal endothelial cells decreased significantly ( $p < 0.01$ ) with increasing concentration of triamcinolone acetonide (fig 1).

For further investigation of the crystalline triamcinolone acetonide effect on neovascular tissue in vivo, an established animal model of retinopathy of prematurity was employed. Cell nuclei counts on the vitreal side of the inner limiting membrane were significantly lower in the study group exposed to high oxygen and treated with intravitreal injection of triamcinolone acetonide than in the oxygen exposed study group without treatment (6.3 (SD 2.1) neovascular cell nuclei per histological section versus 14.95 (5.3) neovascular cell nuclei;  $p < 0.005$ , Mann-Whitney U test) (fig 2). This accounts for a 58% reduction of retinal neovascularisation in the triamcinolone acetonide treatment group. In both study groups, the cell count on the inner limiting membrane was significantly ( $p < 0.001$ , Mann-Whitney U test) higher than in the negative control group. No neovascular cell nuclei were detected on the vitreal side of the inner limiting membrane of the retina in the negative control group.

### DISCUSSION

The proliferation of intraocular vascular tissue in patients with ischaemic retinopathies, such as diabetic retinopathy ROP and ischaemic central retinal vein occlusion is one of the important unsolved problems in clinical ophthalmology. Corticosteroids have been known to reduce inflammation and depending on its concentration, suppress proliferation of cells. Consequently, triamcinolone acetonide has been used for the treatment of many ocular diseases.<sup>4–15</sup> However, it is unclear whether triamcinolone acetonide suppresses the proliferation of retinal endothelial cells directly or indirectly.

The results of this study demonstrated that crystalline triamcinolone acetonide inhibits the proliferation of bovine retinal endothelial cells in vitro. The inhibition was dose dependent. In addition, triamcinolone acetonide suppresses 58% of retinal neovascularisation in an established ROP animal model in vivo. A recent experimental study of Wang *et al* showed that triamcinolone acetonide inhibits the proliferation of choroidal endothelial cells.<sup>23</sup> These combined results suggest that triamcinolone acetonide may be responsible for angiostatic effects in ocular diseases with choroidal and retinal endothelial cell proliferation. This notion is further confirmed by other clinical studies reporting an angiostatic effect of triamcinolone acetonide in patients with age related macular degeneration, diabetic retinopathy and retinal vein occlusions.<sup>10–17 19–24</sup>

It cannot be excluded however that triamcinolone acetonide also suppresses ocular neovascularisation with other mode of actions—that is, through downregulation of vascular endothelial growth factor (VEGF) secreted by the retinal endothelium. Several studies have established the importance of VEGF in the pathogenesis of retinopathy of prematurity.<sup>24–26</sup> It may be possible that triamcinolone acetonide inhibits the expression of VEGF in an ischaemic retina. Nauck and colleagues support this assumption demonstrating an inhibitory effect of corticosteroids on VEGF in pulmonary vascular smooth muscle cells.<sup>27</sup> Recent experiments from our department concur with these findings and indicate that triamcinolone acetonide inhibits expression of VEGF in retinal endothelial cells in a hypoxic retina of a ROP mouse (data not published to date).

Several other substances are under clinical testing for suppression of ocular angiogenesis such as anecortave acetate and anti-VEGF antibodies.<sup>28, 29</sup> The 58% suppression of retinal endothelial cells by triamcinolone acetonide in the concentration employed in the present study is comparable to the other studies using these substances. Anecortave acetate, an angiostatic steroid, inhibits retinal neovascularisation by 50%<sup>28</sup> and the anti-VEGF aptamer achieves a suppression result of 80%.<sup>29</sup>

It still needs to be specified what dosage of triamcinolone acetonide has the highest efficacy and safety. Current clinical research studying the effects of triamcinolone acetonide vary in dose amounts.<sup>10–24</sup> The most frequently tested injected dosages of triamcinolone acetonide are about 4 mg and about 20 mg.<sup>11–22</sup> Results of clinical studies suggest that the efficacy and duration of the effect of intravitreal triamcinolone acetonide are dosage dependent (own data). These clinical results are in agreement with the findings of the present study in which higher dosages of triamcinolone acetonide had a greater effect in suppressing retinal endothelial cell development. On the other hand, too high dosages exhibited cytotoxic effects on intraocular cells as shown in the present study. Clinical randomised studies evaluating the dosage dependency of efficacy and safety of intravitreal triamcinolone acetonide in patients with intraocular oedematous and neovascular diseases will be helpful to clarify this uncertainty.

In conclusion, the present study demonstrated that intravitreal triamcinolone acetonide suppresses the proliferation of retinal endothelial cells *in vitro* and *in vivo*. Triamcinolone acetonide may, therefore, warrant further examination for its intravitreal use as anti-angiogenic therapeutic agent for intraocular neovascular diseases.

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