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Treatment of  
Laser Induced Retinal Injuries

Midterm Report  
(September 30, 1987 through March 31, 1989)

DTIC FILE COPY

N. Naveh, M.D.  
Michael Belkin, M.D.

June 21, 1989

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Tel Aviv University  
Ramat Aviv, Tel Aviv Israel 69978

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<p>Previously we have shown that the ocular response to laser-induced ocular injury is characterized by an enhancement in arachidonic acid metabolism and that steroid treatment curtailed this response. In the present study we investigated the involvement of leukotrienes B<sub>4</sub> (LTB<sub>4</sub>) and the following parameters were studied: LTB<sub>4</sub> production by the retina/choroid, its accumulation in the vitreous, and changes in vitreal protein levels. The effect of an antileukotriene drug on the severity of the ocular inflammatory response was also studied.</p> <p>To overcome the problem that the amounts of LTB<sub>4</sub> produced by the retina in vitro were too small to be detected by the biochemical assay used by us, a model for the study of LTB<sub>4</sub> production by the retina-choroid was established, using Ca<sup>2+</sup> ionophore A 13187. The choice of the antileukotriene drug to be used in our future work was done following an in vitro study in which the efficacy of two antileukotriene drugs were compared (BW755C versus norguaiaretic acid (NDGA)). NDGA has been proven to be the more effective antileukotriene</p>			
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drug. The changes in LTB<sub>4</sub> production and its vitreal accumulation, as well as vitreal protein levels, were studied in eyes exposed to Nd:YAG laser irradiation at various time intervals during a two-week period. Our results confirm our hypothesis, and indicate that the ocular response to laser-induced retinal injury involved an enhancement in chorioretinal LTB<sub>4</sub> production, and its subsequent accumulation in the vitreous to above pre-laser values. It is postulated that LT involvement in the ocular response to trauma might be responsible for the immediate incapacitation and the late vision reduction. Our finding that the ocular response to laser-induced retinal injury involves an enhanced leukotriene B<sub>4</sub> response, might be responsible for edema formation and accumulation of various toxic substances in the vitreous, with resultant immediate incapacitation of the affected individuals. Treatment to reduce leukotriene production might attenuate vision reduction.

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FOREWORD

In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals": prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life and Sciences, National Research Council (NIH Publication, No. 86-23, revised 1985).

Summary

This midterm report covers the work performed from September 30, 1987 through March 31, 1989, and includes data obtained during:

- 1) A period of non-funded, extension (Feb. 87-September 1987).
- 2) Information reported in our recent annual report dated May 1989, covering *work done for the period of 30 Sept. 87 to 1 Oct. 88*.
- 3) Data obtained up to the period of March 31, 1989).

During the period covered by this midterm report we:

- 1) Established a reproducible model for the study of leukotriene B<sub>4</sub> production by retina-choroid.
- 2) Compared the efficacy of two antileukotriene drugs (NDGA and BW755) and chose NDGA as the drug of choice for the treatment of laser injuries.
- 3) Studied changes in leukotriene B<sub>4</sub> levels in the vitreous of laser injured eyes.
- 4) Studied the effect of repeated laser exposure on the leukotriene response.
- 5) Initiated the study on the effect of NDGA on leukotriene B<sub>4</sub> response.

### Introduction

Our study of "Steroid treatment of a laser induced retinal injury" summarized in an annual report (1), demonstrated that Neodymium (Nd):YAG retinal burn was associated with an inflammatory reaction at the laser lesion site; An extensive infiltration by polymorphonuclear cells of both the retina and choroid was associated with excessive production of prostaglandin  $E_2$  ( $PGE_2$ ) by the retina-choroid, which were immediately released into the vitreous causing an increase in  $PGE_2$  vitreal levels to above pre-laser values. The vitreal response involved also an enhanced protein concentration indicative of a "break" in the blood retinal barrier. At later phases after exposure, the injury site underwent scarring.

Steroid treatment of laser exposed animals which was started immediately after laser exposure was only partially effective in reducing the chorioretinal response to trauma, but it significantly curtailed the vitreal reaction; Steroids inhibited altogether the vitreal  $PGE_2$  response but had only a partial inhibitory effect on protein leakage into the vitreous. However, steroid treatment had no effect on the retinal polymorphonuclear cellular infiltration and it suppressed only transiently the excessive  $PGE_2$  production by the damaged choroid-retina.

The fact that steroid treatment was unable to abolish completely the choroid-retina response in eyes subjected to laser irradiation established the need for a search for a better and more effective therapeutic regimen. An effective therapeutic regimen should minimize the ocular response to laser irradiation so that the immediate incapacitation and the chronic scarring process affecting visual rehabilitation will be diminished.



This requirement for a new medication led us to extend our study and look into the behavior of leukotrienes B<sub>4</sub> (LTB<sub>4</sub>) during the ocular response to laser injury.

We hypothesized that laser induced retinal injury might be associated with excessive leukotriene (LT) production. Our hypothesis is based on the fact that the retinal injury was related to an enhanced PGs production which is indicative of excessive availability of free arachidonic acid released from its binding to cellular membrane, which serves as a substrate for LT production (2).

Increased levels of LTB<sub>4</sub> in the aqueous humor of rabbits was demonstrated following ocular trauma (3,4) in uveitis in humans (5). Though no direct correlation was demonstrated between elevated LT levels and the ocular inflammatory response (6).

The significance of LTs in the tissue inflammatory reaction is exemplified in studies in which LT antagonists or LT synthesis inhibitors provided some protection in situations such as endotoxemic shock or various forms of trauma (7,8).

LTs generated in the brain affect cerebral circulation to promote vasospasm and edema (9,10). The retina, which is also a nervous tissue might be affected in a similar manner, so that its exposure to excessive LT levels might have a detrimental effect on vision. Therefore, it must be kept in mind that the laser induced retinal lesion is a pathological process in which both PGs and LTs play a major role.

In order to assess the mediatory role of LT in retinal injury, we suggested to study the effect of two antileukotriene drugs, Norguaiaretic acid (NDGA) and BW755C, on the laser induced retinal injury. NDGA inhibits LT production through inhibition of the lipoxygenase pathway and has no

effect on PGs formation (11). BW755C affects simultaneously the formation of both LTs and PGs (12).

However, in any pathophysiological process where LTs and PGs have been argued to participate, many other biologically active substances including thromboxanes, oxygen radicals, interleukenes, etc. are also produced. Therefore, it is expected that successful therapeutic strategies would include drugs aimed against multiple mediators, in addition to modulation of the LT actions (13).

### Materials and Methods

A total of 192 pigmented rabbits of either sex (2 to 2.5 kg.) were used in this year's study. Rabbits were divided into five study groups: (48, 54, 41, 30 and 21 rabbits in each group, respectively).

- 1) Study on retinal in vitro LTB<sub>4</sub> production.
- 2) Study on the effect of two antileukotriene drugs on LTB<sub>4</sub> in vitro retinal production.
- 3) Study on changes in retinal and vitreal LTB<sub>4</sub> levels of laser exposed eyes.
- 4) Study on repeated laser exposure.
- 5) Study on the effect of NDGA on vitreal LTB<sub>4</sub> of laser exposed eyes.

Animal care and treatment in this investigation were in compliance with the ARVO Resolution on the Use of Animals in Research.

### Laser procedure

Neodymium:YAG laser exposure - A Q-switched Neodymium (Nd):YAG laser (Lasag:Thun, Switzerland) was used to perform 15 retinal laser applications through a Russel-Fankhouser three mirror lense. These applications were aimed at the posterior pole of the right eye of each rabbit nasally to the optic nerve head, at areas devoid of blood vessels, as far apart as possible. The other eye was left untreated. The endpoint in establishing a Nd:YAG laser burn was a visible whitening of the retina at the burn site with slight reddening of the underlying choroid. Excluded were eyes with retinal or vitreal hemorrhage. Each exposure consisted of a single pulse of single burst in the multimode at energy level of 0.1-0.4 millijoules.

Nd:YAG laser exposed group - Rabbits which underwent Nd:YAG laser irradiation were divided into 4 subgroups, studied at 1,3,7 and 14 days after exposure.

Thirty minutes before the laser procedure, the animals were anesthetized by 35 mg/kg Ketamine and 5 mg/kg Xylazine injected intramuscularly following pupil dilatation with Tropicamide 0.5%. Local anaesthesia with Benoxinate 0.1% preceded the laser treatment. Following animals sacrifice, with intravenously injected overdose of phenobarbiturate, the right eye of each rabbit was enucleated, and samples of the vitreous body and the retina-choroid were obtained for  $LTB_4$  and protein measurements as described.

Unexposed control group - Control rabbits were not exposed to any laser irradiation and their vitreous, and retina-choroid of the right eye of each rabbit were obtained for  $LTB_4$  determination as described for the Nd:YAG laser group.

Retina-choroid preparation Following enucleation, the corneas were cut all around the limbus, and the iris and ciliary body were removed by pulling gently at the iris base. The lens was removed and the vitreous expelled as describe (1) and placed separately in a vial. The retina attached to the choroid (a retina-choroid preparation) was separated from the sclera and each preparation was placed in another vial containing the buffer for further studies on LT production, described below.

Leukotriene  $B_4$  determination - The retina-choroid preparation was incubated in 0.6 ml Krebs Ringer Bicarbonate Heppes Buffer, pH 7.4 in a period, the tissue was removed and samples were withdrawn for  $LTB_4$  determination. The vitreous of each eye was similarly incubated following the addition of 0.3 ml of the same buffer. At the end of the incubation period, a sample was withdrawn for  $LTB_4$  determination.  $LTB_4$  was determined using a Radioimmunoassay kit (New England Nuclear) with a specific antibody.

Protein determination - Protein was measured in the vitreous body using the modified Lowry method (13).

Experimental design

Experimental design for the first two groups was similar:

Group 1 - A model for in vitro study of leukotrienes by retina-choroid.

Group 2 - Comparison of the in vitro inhibitory effect of two antileukotriene drugs.

Studying these two subjects involved investigation of the in vitro changes in  $LTB_4$  production by retina-choroid.

In vitro studies - Each retina-choroid preparation was incubated separately for 30 minutes at  $37^{\circ}C$  in a shaking bath in 0.6 ml of a Krebs Hepes Buffer, Ph 7.4 with or without the addition of  $Ca^{+2}$  ionophore A23187 at various concentrations (0.1 to 5.0 micromoles). At the end of the incubation period a sample was withdrawn for  $LTB_4$  determination.

The antileukotriene effect of NDGA and BW755C on chorioretinal  $LTB_4$  production was studied using a retina-choroid model previously described, so that in this set of experiments we used a retina-choroid preparation incubated in  $Ca^{+2}$  ionophore containing media. The appropriate drug was also added to the incubation medium using various doses (see results section). At the end of a 30 minute incubation period, samples were withdrawn for  $LTB_4$  determination. The amounts which accumulated in the incubation medium containing the retina-choroid was considered as representing the amounts produced by the tissue, and this was dubbed - " $LTB_4$  in vitro production".

Group 3. Changes in retinal and vitreal  $LTB_4$  response in laser injured eyes

This was studied in vivo, in two groups of rabbits; an unexposed control group and a laser exposed group. In the laser exposed group, the retina-choroid was obtained at 1,3,7, and 14 days after exposure and then

incubated, as described. Samples from the incubation media containing the retina-choroid were withdrawn for determination of  $\text{LTB}_4$  in vitro production while samples from the vitreous were used for determination of vitreal protein and  $\text{LTB}_4$  levels.

Group 4. Effect of repeated laser exposure on  $\text{LTB}_4$  vitreal response - Animals were lasered in a manner similar to that used in groups 3 and 5 and two weeks later were lasered again, and this time the laser applications were aimed at areas free of visible laser lesions. The animals were sacrificed at 1,3,7 and 14 days after the second laser exposure.

Group 5. The in vivo effect of an antileukotriene drug (NDGA) on the vitreal  $\text{LTB}_4$  response in laser exposed eyes - Laser procedure followed that used in groups 3-5, but within 1 hour after exposure NDGA was administered intramuscularly, and this treatment was repeated daily.

## Results

### Group 1 - Study of the in vitro chorio-retinal LTB<sub>4</sub> production.

LTB<sub>4</sub> in vitro production by the choroid-retina yields amounts which are too small to be detected by the biochemical assay used by us. Therefore, measure to enhance retinal LTB<sub>4</sub> in vitro production were required.

Production was enhanced by adding Ca<sup>+</sup> ionophore A13187, a drug widely used as an activator of LTB<sub>4</sub> production. The effect of varied concentrations of Ca<sup>++</sup> ionophore was studied (from 0.1 to 5.0 micromoles) in 48 rabbits divided into 6 groups (Table 1).

The addition of Ca<sup>+</sup> ionophore (0.1 to 1.0 micromoles) caused a dose dependant increase in retinal LTB<sub>4</sub> production (Table 1), and maximal in vitro production levels were achieved at 1 micromole. With higher levels of Ca<sup>+2</sup> ionophore production was enhanced to a lesser degree and at 5 micromolar no excitatory effect was noted. On the addition of 1 micromole Ca ionophore LTB<sub>4</sub> in vitro production levels reached levels of 389±132 pg/gm wet weight (Table 1) which were easily detected by our biochemical assay. We find this method of using Ca ionophore for measurement of in vitro chororetinal LTB<sub>4</sub> production to be a satisfactory model.

### Group 2 - Comparison of the in vitro efficacy of two antileukotriene drugs (BW755C and NDGA).

The study of the inhibitory effect of the two antileukotriene drugs - BW 755C and NDGA on LTB<sub>4</sub> in vitro production by the choroid-retina was carried out to decide their relative efficacy. The drug possessing a greater inhibitory effect, is likely to be more active as an antiinflammatory agent.

Comparison of the two antileukotriene drugs involved 54 rabbits and was performed in a dose dependant manner. Each drug was studied at 3 different concentrations (0.1, 1.0 and 10 micrograms/ml). The appropriate drug was added separately to a vial containing a single retina choroid incubated in a media with  $\text{Ca}^{+2}$  ionophore (1.0 micromole). Following a 30 minute incubation period in  $37^{\circ}\text{C}$  in a shaking bath, a sample from the media was withdrawn for  $\text{LTB}_4$  determination.

In evaluating our results, we considered as baseline the amounts of  $\text{LTB}_4$  produced by choroid-retina treated with  $\text{Ca}^{+2}$  ionophore only, but with no other drug added ( $352 \pm 128$  per/gr. wet wt.). Both BW755C and NDGA had a significant inhibitory effect on  $\text{LTB}_4$  production which was evident at each of the concentrations studied (Table 2).

NDGA at low concentration (0.1 microgm/ml) inhibited baseline  $\text{LTB}_4$  production by 90% and was more effective than the corresponding BW755C group ( $27 \pm 13$  and  $107 \pm 30$  pg/gm wet wt., respectively). At higher dose (1.0 microgm/ml) both drugs exhibited a similar effect; demonstrating an inhibitory effect which reduced levels to less than 10% baseline values. The standard deviation in the two latter groups were equal or greater than the mean and this was caused by the fact that production ranged from 0 to 40. Based on our data, we decided to use NDGA as our drug of choice for inhibition of  $\text{LTB}_4$  production in our animal studies.

Group 3 - Changes in vitreal and retinal  $\text{LTB}_4$  in laser injured eyes.

The determination of  $\text{LTB}_4$  in the choroid-retina and vitreous of eyes subjected to Nd:YAG laser irradiation encountered two main problems: The problem of the naturally low leveled retinal production was overcame by the addition of  $\text{Ca}^{+}$  ionophore A13187 to the media of the incubated retina, following the method described in "Results", section A.



The second problem of low vitreal  $LTB_4$  levels was solved by increasing the number of laser application to 15 using the same power setting (method section, laser procedure).

Baseline (pre-laser) vitreal content of  $LTB_4$  was very low and ranged from 0 to 33 pg/gm weight (wt) with an average of  $19 \pm 9$  pg/gm wt. Following laser exposure, vitreal  $LTB_4$  levels were elevated to above pre-laser values and peaked on day 3, at which time they were 280% higher than pre-laser values (Figure 1).

Nd:YAG laser induced retinal damage was associated with an enhanced chorioretinal  $LTB_4$  production throughout the first week following exposure. An augmented production (200% higher than baseline) was evident already at the first day, and remained elevated also on day 3. Maximal production values were achieved on day 7 and reached levels 2.7 times higher than pre-laser values. Control levels were resumed on day 14.

#### Group 4 - Repeated laser exposure.

This study on exposure to repeated laser irradiation might be of military significance; Laser exposure to low energy levels, might go unnoticed during the initial phase (if the lesion site does not include the macular area). Therefore, the unaware soldier will be sent back to combat where he might be exposed to additional laser irradiation. We suggested that a repeated laser exposure might result in an accumulative damage to the blood retinal barrier, similar to that described by Bito (1974 with a progressive accumulation of toxic substances in the vitreous body and a subsequent reduction in visual acuity.

In the present series of experiments, the animals were lased once and two weeks later were exposed to a second similar session of laser irradiation. During the second laser exposure, the laser was aimed at areas

free of visible laser lesions (as described under experimental design). Following the second laser exposure, the animals were sacrificed and Leukotriene B<sub>4</sub> was determined in the media containing the chorioretina or the vitreous of each eye, while protein was measured only in the vitreous as described.

Our results (Fig. 2) demonstrate that repeated laser exposure is related to an increase in LTB<sub>4</sub> vitreal concentrations to above pre-laser values. Peak levels, 7 and 4.5 folds higher than baseline were noted during the second week after exposure (days 7 and 14 respectively), while earlier levels did not exceed pre-laser. The pattern of changes of vitreal LTB<sub>4</sub> content in eyes subjected to repeated exposure was different from that observed in eyes exposed to the laser only once; In the latter group, vitreal LTB<sub>4</sub> values peaked earlier after exposure and maximal levels were lower. Later during the second week baseline levels were resumed (Fig. 2).

This pilot study serves as a partial confirmation to our suggestion that repeated laser exposure to low energy levels might be associated with a more pronounced vitreal response than that observed in eyes exposed to laser only once. This enhanced LTB<sub>4</sub> response occurring late after exposure, might indicate that repeated laser exposure could be unexpectedly harmful to the eye.

#### Group 5 - Effect of NDGA on laser injury.

In this group we tried the effect of an antileukotriene drug - NDGA on the ocular inflammatory response following laser exposure. The parameters studied were: the changes in LTB<sub>4</sub> in vitro production by the choroid-retina and its accumulation in the vitreous.

The decision as to which antileukotriene drug should be used as an effective antiinflammatory agent, was based on our previous work (see group

2). Our data on the comparison between two antileukotriene drugs; the BW755 and NDGA showed the supremacy of the NDGA. Therefore, in our study on the efficacy of an antileukotriene drug in reducing the ocular response to laser injury we used NDGA. The experimental design followed that already was described and involved exposure to the Nd:YAG laser and NDGA was given intramuscularly within 1 hour following injury and daily during the two week observation period. The exposed animals were sacrificed at 1,3, and 7 days after exposure, the eyes were enucleated and samples of retina-choroid, and vitreous body were obtained.

In assessing our results, the pre-laser values were considered as baseline (100%) and levels in the treated and untreated groups were given as percentage of baseline. Our results on the effect of NDGA treatment on the vitreal  $LTB_4$  response in laser exposed eyes (Fig. 3) indicate that NDGA reduced the accumulation of  $LTB_4$  in the vitreous during the first week after exposure.

The inhibitory effect of NDGA persisted for a whole week, thus protecting the retina from the adverse effect of excessive levels of  $LTB_4$ .

Conclusive remarks

The findings obtained during this period study which have a bearing on future studies are:

- 1) The newly developed method for determination of retinal  $LTB_4$  production, which otherwise was undetectable, is going to serve us in the coming two years. This method is reproducible and might serve for analysis of the antiinflammatory properties of various drugs.
- 2) Our finding that NDGA, an natileukotriene drug caused a 90% inhibition of LT synthesis by the retina, made us choose it as a treatment to reduce to laser induced response in our future in vivo studies.

Military Clinical Significance

Our finding that the ocular response to laser induced retinal injury involved an enhancement in chorioretinal  $LTB_4$  production and its subsequent accumulation in the vitreous has not been reported so far, and has direct clinical implications.

LTs involvement in the ocular response to trauma might be responsible for edema formation and accumulation of various toxic substances in the eye affecting the retina, with resultant immediate incapacitation of the affected individuals. Measures to inhibit LT production might reduce attenuate the scarring process so that vision reduction will be minimized.

In addition our finding that repeated laser exposure is related to a more pronounced vitreal inflammatory response is of significance in cases in whom the first laser exposure went unnoticed. Is a soldier will be prone to another, more harmful exposure which might have a long lasting effect on his vision.

Our finding that NDGA, an antileukotriene drug suppresses, the vitreal  $\text{LTB}_4$  response is promising and might indicate its potency as an antiinflammatory agent with resultant protective effect in laser injury. Further studies are required to substantiate this.

Table 1

The effect of  $\text{Ca}^{++}$  ionophore A23187 on the in vitro retinal production of Leukotriene  $\text{B}_4$ .

$\text{Ca}^{++}$ ionophore Concentration (micromole)	0.1	0.3	0.5	1	2	5
Retinal Leukotrienes levels (pg/gr wet weight) (mean $\pm$ SD)	123 $\pm$ 50	316 $\pm$ 60	285 $\pm$ 134	389 $\pm$ 132	93 $\pm$ 76	0
number of eyes studied	8	8	8	8	8	8
*Control Leukotriene Levels pg/gr wet wt.	12 $\pm$ 7					

\*Retinal leukotrienes production by retina-choroid untreated by  $\text{Ca}^{++}$  ionophore.

Table 2

A comparison of the inhibitory effect of two antileukotriene drugs (BW 755C and NDGA) on leukotriene B<sub>4</sub> production by the retina-choroid.

\* Leukotriene B<sub>4</sub> production by the retina-choroid (pg/gr wet wt) Mean ± SD

<u>Concentration</u> (microgram/ml)	<u>BW 75</u>	<u>NDGA</u>
0.1	107±30 n=9	27±13 n=9
1.0 microgram/ml	35±64 n=9	23±20 n=9
10 microgram/ml	0 n=9	0 n=9
Control	352±128 n=6	

Table 3

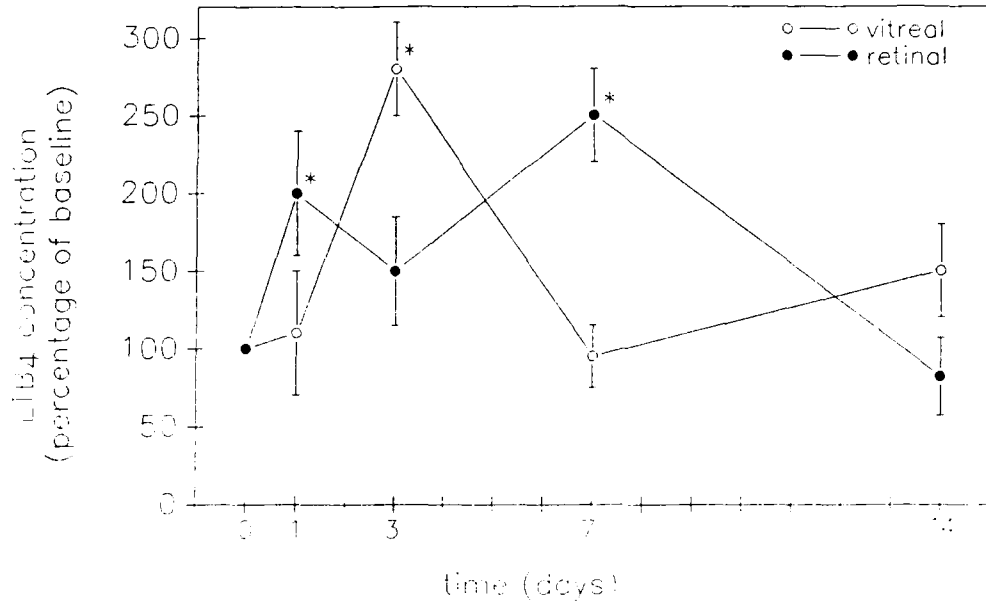
Changes in vitreal and Retinal leukotrienes B<sub>4</sub> following Neodymium:YAG laser induced retinal injury.

Time (days)	Vitreal LTB <sub>4</sub> pg/gr wt. (mean±SD)	Retinal LTB <sub>4</sub> production pg/gr wet wt. (mean±SD)
1	28±14 n=5	390±104 n=5
3	79±31 n=6	307±99 n=6
7	24±15 n=4	575±228 n=6
14	35±13 n=4	174±29 n=5



Fig. 4

Leukotriene B<sub>4</sub> in the Vitreous and its in vitro production by the Retina-Choroid



\* Statistically significant difference from baseline

Figure 2.

Vitreous Leukotriene B<sub>4</sub> concentrations  
following repeated Laser exposure

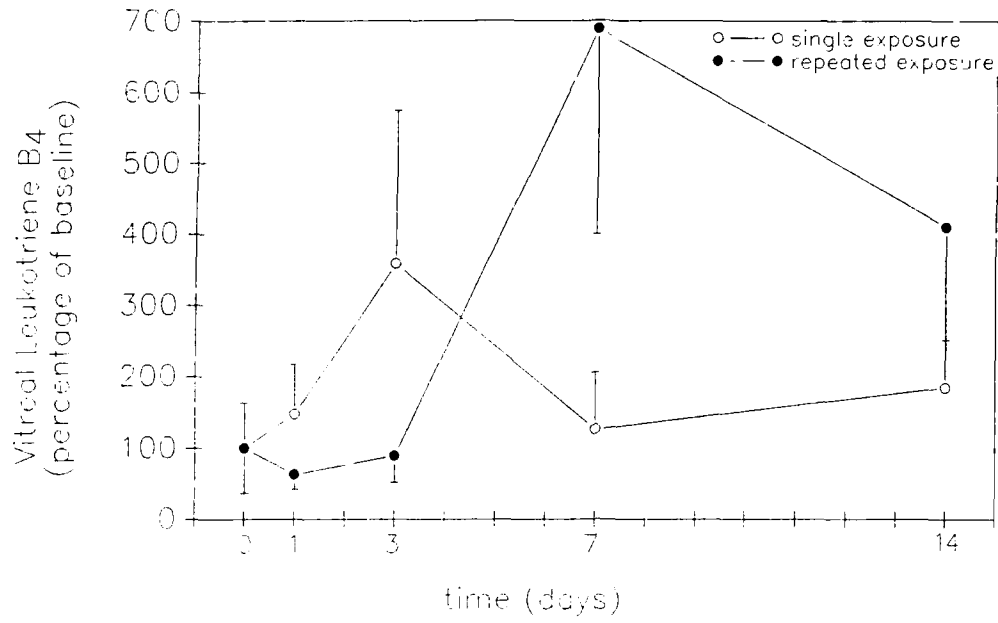
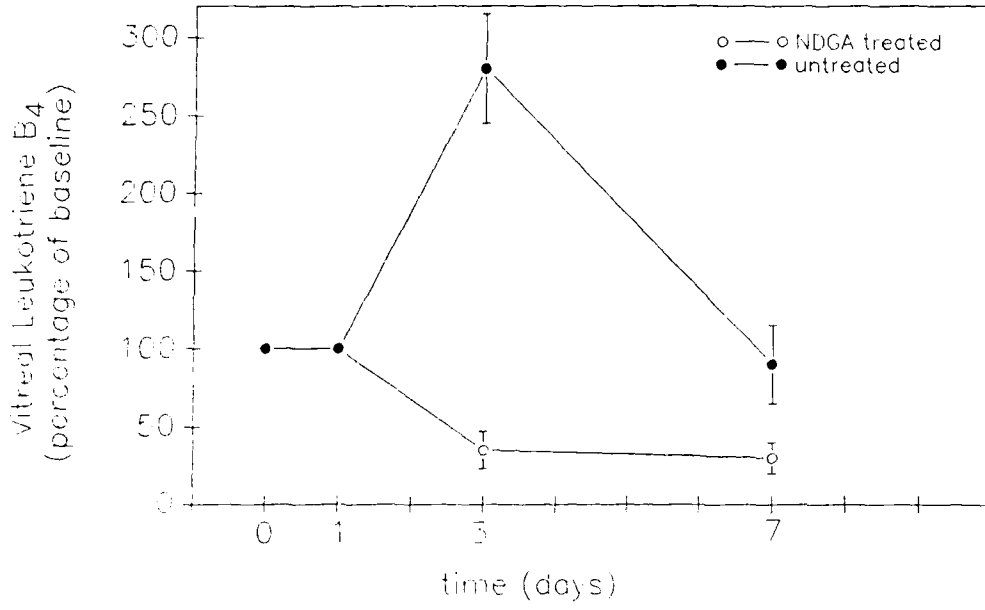


Fig. 3

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Vitreous Leukotriene B<sub>4</sub> concentration  
effect of NDGA



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