Research article

In-vivo inhibition of latanoprost induced iridal hyperpigmentation in rabbits- An investigational study

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ABSTRACT

Objectives: To evaluate the inhibitory effects of different concentrations of α-methyl-DL-tyrosine on latanoprost-induced iridal hyperpigmentation in rabbits.

Methods: We investigated 4 groups of rabbits. Both eyes of the pink, red, and blue groups were treated with latanoprost followed by 0.5%, 1%, and 2% α-methyl-DL-tyrosine (inhibitor) in the right eyes respectively and the green group received only inhibitor. We prospectively investigated the irides, estimated quantitatively total melanin contents, and studied any histopathological changes that occurred.

Results: The observers favored hyperpigmentation in the left eyes while in the right eyes they noted a decrease in pigmentation as compared to the baseline. An increase in pigmentation was noted by 93.33% of observers in the left eye of the blue group. A significant difference in the mean melanin contents was noted in the blue group (Right eye = 0.9560 μg/g (±0.750), Left eye = 3.730 μg/g (±1.062). There was no evidence of stromal malignant changes, Hemorrhage, mitosis, inflammation, and atypical melanocytes in all specimens. A moderate degree of pigmentation in the left eye of the red group was noted. Mild stromal-free melanin pigment was present in all samples of pink, red and blue groups.

Conclusions: The α-methyl-DL-tyrosine significantly inhibited latanoprost-induced iridal pigmentation without causing any histopathological changes at a 2% dose.

1. Introduction

Latanoprost is widely used to treat glaucoma worldwide. It is an F2α prostaglandin analog marketed in 1996, with marked efficacy to reduce intra-ocular pressure up to 35% at a once-daily dose in the evening, and still the drug of choice. Its mode of action is believed to increase uveoscleral outflow by modifying the extracellular matrix [1].

Latanoprost is well tolerated generally in all patients. Published safety data indicates several side effects like conjunctival hyperemia, iridal hyperpigmentation, hypertrichosis of eyelashes, and darkening of peripheral areas. Ocular inflammation, delayed anterior uveitis and cystoid macular edema are other rare side effects reported with the use of latanoprost [2].

Amongst other side effects increase in iridal pigmentation got the special interest of scientists and it is reported in the United States (12%), United Kingdom (22.9%), and Scandinavian (10.8%) clinical studies. This hyperpigmentation is theoretically linked with increased melanogenesis [3, 4, 5]. The increase in pigmentation was first evidenced in phase III clinical trials and darker color was exhibited in irides of patients treated with latanoprost after 3–6 months [6]. A study conducted in Sweden revealed that this concentric increase in the iridal pigmentation starts after 6 months of treatment with latanoprost and was found in 2/3 of patients. They also observed that this change in iris color was permanent because there was no reversal of color after the cessation of treatment [7]. Besides human studies, latanoprost-induced iridal hyperpigmentation was also observed in many animal model studies like in 9/11 rabbits, and heterochromia was observed in a study conducted in New York [8].

However, it is a matter of concern whether this hyperpigmentation may have any harmful effects or is just a cosmetic effect of heterochromia.
when latanoprost is used unilaterally to treat glaucoma. Another valid concern is that, whether this increased pigmentation can also affect the outflow pathways which may eventually cause blockage or lead to pigmentary glaucoma [9]. Many drugs can bind to the melanin contents of the eye and previous studies also support the altered pharmacokinetics and/or pharmacodynamics of several drugs like ofloxacin, pefloxacin, gentamicin, acyclovir, celecoxib, etc. due to the presence of melanin pigment [10].

Keeping in view the scenario of irreversible hyperpigmentation and valid concerns about harmful effects, very limited work was done on the inhibition of iridal pigmentation induced by latanoprost. An in-vitro study conducted by DRAGO F et al revealed that α-methyl-para-tyrosine (Tyrosinase inhibitor) inhibited melanin synthesis in cultured melanocytes when used concomitantly with latanoprost [11]. Their experiments were based on the usage of cultured cells and limitations of the in-vitro design of the study, we decided to conduct an in-vivo study in rabbits by using the same inhibitor along with latanoprost with the objectives to evaluate the inhibitory effects of different concentrations of α-methyl-DL-tyrosine on latanoprost induced iridal hyperpigmentation.

We prospectively investigated the irides by a panel of masked observers, estimated quantitatively total melanin contents, and studied any histopathological changes that occurred in the irides by the use of both drugs as well as any change in the physical appearance of eyes. As per our knowledge, it is the first in-vivo study of its type in the world, especially in Pakistan. It will open a new door of research and a line for the Pharmaceutical industry to produce such innovative products to treat glaucoma effectively without any concern of hyperpigmentation.

2. Materials and methods

This investigative study was carried out using rabbits at the Pharmacy department, University of Malakand, Chakdara Pakistan from April 2021 to October 2021 after taking formal permission from the departmental research ethics committee (DREC) on 13th April 2021, Vide reference NO: 1373. Young and healthy rabbits (12) of average weight (1.85 kg) were randomly and equally divided into 4 groups and kept in the natural environment to eat and drink normally. Each group was differentiated by painting the inner ear of each rabbit with a special color indicating the group name (Pink, Red, Blue, and Green) based on the different concentrations (0.5%, 1%, and 2%) of inhibitor and a control. Latanoprost 0.005% ophthalmic solution (Commercially available brand) as pigmentation inducer and α-methyl-DL-tyrosine (Obtained from Sigma Aldrich, Germany) as inhibitor were used.

Both eyes of the pink, red, and blue groups were treated with 1 drop of latanoprost in the evening followed by 1 drop of 0.5%, 1%, and 2% α-methyl-DL-tyrosine in the right eyes respectively, 5 min after the use of latanoprost. Left eyes were treated as a control in these groups. The green group (control) received only α-methyl-DL-tyrosine (inhibitor), 0.5% and 2% in the left and right eyes respectively. This study was continued for the period of six months and photographs of each eye were taken by a still camera (Canon 400D DSLR, Japan with kit lens 18–55 mm (Magnification) at a distance of approximately 12 inches, without filter, and a Shutter speed was 150) at 0 (baseline) and 6th months. During the study period, all of the rabbits were examined for their bi-monthly routine eye checkup by an expert ophthalmologist, and the occurrence of any changes was noted as mild (+), moderate (++), and heavy (+++). On completion of the study period, all of the rabbits were humanly sacrificed by holding them from their hind limbs with the left hand while hanging down and applying a smart blow with the right hand at the back of the neck to cause cervical dislocation. After a while, the irises were removed (Figure 1) by an expert eye surgeon and stored in 10% neutral buffered formalin solution separately in sample bottles. The isolated irises were evaluated for quantitative estimation of total melanin contents and histopathological studies.

2.1. Evaluation of irides photographs by observers

The irides photographs were evaluated by displaying magnified views of slides from a projector and a panel of masked observers (four Glaucoma Specialists and one Histopathologist) were asked individually to determine whether there was any progression of the pigmentation by comparing the photographs displayed and noting their observations on the given proforma without discussing with each other. The pigmentation was classified into no change, pigmentation increased or decreased. The comparison was made between photographs taken at 0 months (baseline) and the 6th month of each eye and between the left and right eye of each rabbit. Results were calculated in percentages by applying the following formula.

![Image](image1.jpg)

Figure 1. Isolation of iridal specimens from Rabbit's eye by surgeon ophthalmologist.
2.2. Quantitative estimation of total melanin contents

The total iridal melanin contents were measured by using the sodium hydroxide solubilization method. The tissues were weighed by using the sensitive electronic digital balance (Sartorius) in a controlled environment in tubes (Eppendorf, Fremont, CA). After adding 100 μL of 1 M NaOH (pH 12) and 10 μL of dimethyl sulfoxide (DMSO), samples were boiled for 30–45 min to solubilize melanin. Then samples were brought up to 500 μL with distilled water and were neutralized by using diluted acetic acid (1 M). The absorbance of the samples was then measured at 475 nm by using a spectrophotometer (UV-530 Jasco) against the blank buffer immediately after the solubilization of melanin. The total melanin content was quantified in the tissues by using synthetic melanin standards (Obtained from Sigma Aldrich, Germany) processed with a method similar to that used for the tissue samples [10].

2.3. Histopathological evaluation of irides

The formalin-fixed tissue specimens were sent to histopathologists with no knowledge of iridal treatment and asked for routine examination with a special focus on melanocytes and melanin granules, degree of anterior border layer Pigmentation, Number of stromal pigmented melanocytes (To be expressed as cells/HPF), Stromal cell pigmentation, Stromal free melanin pigment, Stromal inflammation level, malignant changes in stromal blood vessels, Hemorrhage (Stroma or anterior layer), Stromal mitosis (H&E, without K167) and atypical melanocytes. The specimens were graded by level of pigmentation and degree of inflammation in different cells as none, mild, moderate, and dense (Table 1). The observations were recorded on histology grading forms and calculations were expressed in percentages by using the same formula mentioned above.

2.4. Statistical analysis

All of the data was evaluated by using SPSS (Inc. Released 2008. SPSS Statistics for Windows, Version 18.0. Chicago: SPSS Inc.) Software. The frequencies of all variables were calculated and represented in the form of tables and figures. For quantitative variables mean and standard deviations (SD) were calculated to represent the central tendency and dispersion. For the comparison of groups, one-way ANOVA was used. All statistical tests were taken significantly at p-value < 0.05.

3. Results

3.1. General observations

During the study period, although almost all of the rabbits were found healthy, active, and normal few female rabbits gave birth to premature babies in latanoprost-treated groups. In the green group lethargy, lack of appetite, and rough dry skin was noted in 2 out of 3 rabbits, and infrequent ataxia in 1 rabbit after the 4th month of the study.

During bi-monthly routine eye checkups (Table 3), the conjunctiva of the majority of rabbits was normal except in a few, occasional mild hyperemia was noted. Corneal haziness was present in a few eyes whereas, increased iridial pigmentation was observed in left eyes in a few rabbits after 4 months, especially in red and blue groups. Pupil size was 6–7 mm and intraocular pressure (IOP) was within the normal range (up to 20 mmHg) generally in all rabbits.

3.2. Evaluation of irides photographs by observers

The outcome of photographs of each eye taken on the 1st day (0 months) of the study (baseline) and after the completion of 6 months are shown in Table 3(a) and Figure 2. In left eyes (treated with latanoprost alone) of both pink and red groups, 66.67 % and in the blue group 80% of observers favored hyperpigmentation in 6th month as compared to baseline. On the other hand in the right eyes (treated with both latanoprost and inhibitor), 53.33% of observers declared no change in the pink group while 26.67% & 73.33% in red and blue groups respectively noted a decrease in pigmentation. In the green group, no change was observed by 60% of observers in the left eye while 66.67% noted a decrease in pigmentation in the right eye. In Table 3(b) and Figure 3, an increase in pigmentation was declared by 93.33% of observers in the left eye as compared to the right eye of the blue group while 66.67% noted no change in pigmentation in both eyes of the Green group at 6th month of the study.

3.3. Quantitative estimation of total melanin contents

The quantitative estimation of total melanin contents (μg/g of sample and percent melanin contents) of isolated iris samples of rabbits (all groups) after treatment with latanoprost and α-methyl-DL-tyrosine is exhibited in Table 4. There was no difference in the mean melanin contents (μg/g of sample) of the left (10.860 (±0.695)) and right (10.765 (±0.622)) eyes of the pink group. A slight difference was observed in mean melanin contents in red group (Right eye = 14.894 μg/g (±1.210), Left eye = 16.043 μg/g (±1.331)) while a significant difference (p-Value = 0.022) was noted in blue group (Right eye = 09.560 μg/g (±0.750), Left eye = 13.730 μg/g (±1.062)). The green group also showed the almost same amount of melanin content (μg/g) in both eyes.

3.4. Histopathological evaluation of irides

The results of specimen grading examined by masked observers (Histopathologists) to study any histological changes and abnormalities present are shown in Table 5 and Figure 4. There was no evidence of malignant changes in stromal blood vessels, Hemorrhage of the stromal or anterior layer, stromal mitosis, and atypical melanocytes in all 24 specimens (right & left eyes of all groups). A mild degree of anterior border layer pigmentation was found in 66.7–100% of specimens in the right eyes (treated with both latanoprost and inhibitor) of all groups as compared to the left eye (treated with only latanoprost) of the red group where it showed a moderate degree of pigmentation in 66.7% (2/3) of

| Table 1. Summary of grading, pigmentation level, and degree of inflammation in isolated specimens of rabbit’s irides treated with latanoprost (Drug), α-methyl-DL-tyrosine (Inhibitor), or both. |
|-------------------------------------|-----------------|
| The degree of pigmentation in different cells | Pigmentation grade |
| No pigmented cells/HPF               | None            |
| 1-2 cells/HPF                       | Mild            |
| 3-4 cells/HPF                       | Moderate        |
| >4 Cells/HPF                        | Heavy           |

| Stromal free pigment levels |
|----------------------------|
| Percentage of the total tissue available showing free pigment i.e. pigment outside the cells | Stromal free pigmentation grade |
| <20% areas showing free pigment | Mild |
| 21–50% areas showing free pigment | Moderate |
| 51–100% area showing free pigment | Heavy |

| Stromal inflammation level |
|----------------------------|
| Pattern of inflammatory cells present | Degree of inflammation |
| Scattered cells              | Mild |
| Single cluster of cells or single lymphoid follicle formation | Moderate |
| More than single cluster or single lymphoid follicle | Dense |
| No inflammatory cells        | None |

| Table 2. Comparison of irides photographs by observers (number of observers agreed (N) vs. total number of observers (T)) |
|---------------------------------------------------------------|
| Observations (Outcome)/Total number of observations          |
| Number of observations (Outcome)/Total number of observations |

| | Left eye | Right eye |
|---|-----------|-----------|
| No pigmented cells/HPF | None | None |
| 1-2 cells/HPF | Mild | Mild |
| 3-4 cells/HPF | Moderate | Moderate |
| >4 Cells/HPF | Heavy | Heavy |

| | Left eye | Right eye |
|---|-----------|-----------|
| <20% areas showing free pigment | Mild | Mild |
| 21–50% areas showing free pigment | Moderate | Moderate |
| 51–100% area showing free pigment | Heavy | Heavy |

| | Left eye | Right eye |
|---|-----------|-----------|
| Scattered cells | Mild | Mild |
| Single cluster of cells or single lymphoid follicle formation | Moderate | Moderate |
| More than single cluster or single lymphoid follicle | Dense | Dense |
| No inflammatory cells | None | None |

| Table 3. Comparison of irides photographs by observers (number of observers agreed (N) vs. total number of observers (T)) |
|---------------------------------------------------------------|
| Observations (Outcome)/Total number of observations          |
| Number of observations (Outcome)/Total number of observations |

| | Left eye | Right eye |
|---|-----------|-----------|
| No pigmented cells/HPF | None | None |
| 1-2 cells/HPF | Mild | Mild |
| 3-4 cells/HPF | Moderate | Moderate |
| >4 Cells/HPF | Heavy | Heavy |

| | Left eye | Right eye |
|---|-----------|-----------|
| <20% areas showing free pigment | Mild | Mild |
| 21–50% areas showing free pigment | Moderate | Moderate |
| 51–100% area showing free pigment | Heavy | Heavy |

| | Left eye | Right eye |
|---|-----------|-----------|
| Scattered cells | Mild | Mild |
| Single cluster of cells or single lymphoid follicle formation | Moderate | Moderate |
| More than single cluster or single lymphoid follicle | Dense | Dense |
| No inflammatory cells | None | None |
Table 2. Summary of bimonthly ocular examination of rabbits by a specialist ophthalmologist to note any changes that occurred during the study period in all groups. The changes were noted as mild (+), moderate (++), and heavy (+++).

| Group | Check Points | Right Eye | Left Eye | Right Eye | Left Eye | Right Eye | Left Eye |
|-------|--------------|-----------|---------|-----------|---------|-----------|---------|
|       | Eye Examination | After 2 months | After 4 months | After 6 months | After 2 months | After 4 months | After 6 months |
| Pink 1 | Conjunctiva | Normal | Normal | Normal | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 6 mm | 6 mm | 7 mm | 6 mm | 6 mm | 6 mm |
|       | IOP | 15 | 16 | 18 | 18 | 17 | 15 |
| Pink 2 | Conjunctiva | Normal | Normal | Normal | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | ++ | - | - | - | - |
|       | Pupil Size | 5 mm | 6 mm | 6 mm | 6 mm | 6 mm | 7 mm |
|       | IOP | 13 | 15 | 12 | 14 | 15 | 15 |
| Pink 3 | Conjunctiva | Normal | Normal | Normal | Hyperemia | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 7 mm | 7 mm | 6 mm | 7 mm | 7 mm | 7 mm |
|       | IOP | 17 | 16 | 16 | 13 | 15 | 15 |
| Red 1 | Conjunctiva | Normal | Hyperemia | Normal | Normal | Normal | Hyperemia |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 6 mm | 6 mm | 6 mm | 6 mm | 7 mm | 7 mm |
|       | IOP | 15 | 14 | 13 | 16 | 17 | 17 |
| Red 2 | Conjunctiva | Normal | Normal | Normal | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 6 mm | 5 mm | 5 mm | 5 mm | 6 mm | 6 mm |
|       | IOP | 15 | 17 | 15 | 15 | 13 | 16 |
| Red 3 | Conjunctiva | Hyperemia | Hyperemia | Normal | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 5 mm | 5 mm | 5 mm | 5 mm | 6 mm | 6 mm |
|       | IOP | 15 | 17 | 15 | 15 | 13 | 16 |
| Blue 1 | Conjunctiva | Hyperemia | Normal | Normal | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 7 mm | 6 mm | 7 mm | 7 mm | 7 mm | 6 mm |
|       | IOP | 16 | 16 | 17 | 13 | 14 | 16 |
| Blue 2 | Conjunctiva | Normal | Normal | Hyperemia | Normal | Hyperemia | Normal |
|       | Cornea | Clear | Clear | Haze | Clear | Haze | Clear |
|       | Iris | + | - | + | - | - | - |
|       | Pupil Size | 6 mm | 6 mm | 6 mm | 6 mm | 7 mm | 6 mm |
|       | IOP | 17 | 16 | 17 | 16 | 19 | 15 |
| Blue 3 | Conjunctiva | Normal | Normal | Normal | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 7 mm | 7 mm | 6 mm | 6 mm | 7 mm | 6 mm |
|       | IOP | 18 | 16 | 17 | 14 | 15 | 18 |
| Green 1 | Conjunctiva | Normal | Hyperemia | Normal | Hyperemia | Hyperemia | Hyperemia |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 7 mm | 7 mm | 7 mm | 7 mm | 6 mm | 6 mm |
|       | IOP | 17 | 15 | 20 | 15 | 18 | 15 |
| Green 2 | Conjunctiva | Normal | Normal | Normal | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 7 mm | 7 mm | 8 mm | 8 mm | 8 mm | 8 mm |
|       | IOP | 15 | 13 | 17 | 15 | 18 | 18 |
| Green 3 | Conjunctiva | Normal | Normal | Hyperemia | Normal | Normal | Normal |
|       | Cornea | Clear | Clear | Clear | Clear | Clear | Clear |
|       | Iris | - | - | - | - | - | - |
|       | Pupil Size | 7 mm | 7 mm | 8 mm | 8 mm | 8 mm | 8 mm |
|       | IOP | 15 | 13 | 17 | 15 | 18 | 18 | (continued on next page)
specimens. A mild number of stromal pigmented melanocytes (cells/HPF) were found in 66.7–100% of specimens in the right eyes of all groups except the green group (treated only with inhibitor) where none of the stromal pigmented cells were present in 66.7% (2/3) of iris specimens. While left eyes (treated with only latanoprost) showed the presence of stromal pigmented melanocytes in 100% (3/3) of iris specimens in pink and blue groups. Stromal cell pigmentation was present in 100% (3/3) of left eye iris specimens of the blue group followed by Red and pink left eye samples (66.7%). Mild stromal free melanin pigment was present in 100% of samples (Right, Left eyes) of pink, red and blue groups while in the green group it was in 66.7% (2/3) of irises. There was no stromal inflammation was found in all groups (left & right eyes) except pink where a mild inflammation was noted in 33.3% (1/3) samples.

4. Discussion

4.1. General observations

During the whole study period, we generally observed all rabbits for their eating behavior, activeness, and physical appearance. For their ocular health, a bimonthly comprehensive routine eye checkup by a specialist ophthalmologist was arranged. In our present study, we observed abortions several times in a few rabbits especially those treated with latanoprost. This was a possible outcome as evident in previous observations (n – 6th Month) but in red and blue groups where 1% and 2% inhibitor was used respectively, the difference in mean melanin contents (right vs left eyes) was observed (right vs left eyes) in the mean melanin contents (μg/g of sample) but in red and blue groups where 1% and 2% inhibitor was used respectively, the difference in mean melanin contents (right vs left eyes) was noted. It means by the increase in doses of inhibitor, the inhibition of latentoprost-induced pigmentation was started and at the dose of 2% of inhibitor (Blue group), significant inhibition was observed (Right eye = 09.560 μg/g (±0.750), Left eye = 13.730 μg/g (±1.062)).

4.2. Evaluation of irides photographs by observers

In the current observer’s masked study 93.3% favored pigmentation increased in the left eye as compared to the right eye in the blue group. It means latanoprost treated eye showed more pigmentation than in the eye where α-methyl-DL-tyrosine (Inhibitor) was concomitantly used with latanoprost and α-methyl-DL-tyrosine showed this inhibition at the dose of 2% in the blue group. These observations were also consistent with the quantitative estimation of total melanin contents in the blue group, mean melanin contents (μg/g of sample) were higher in the left eye as compared to the right eye. On the other hand, a dose-dependent decrease in pigmentation as compared to baseline was noted in the right eyes using inhibitor alone or along with latanoprost. It means α-methyl-DL-tyrosine, not only inhibited latanoprost-induced hyperpigmentation but also a natural synthesis of melanin in treated irides. Although it was the outcome of the observer’s findings but can lead to a fear of iridal discoloration on long-term use. As it was the first study of its type (In-vivo inhibition) so we can’t compare the results with other previous studies. However, there are multiple studies available that have been described to determine the optical properties of tissues (including human irides), as it is important to develop models to describe light propagation within tissues and melanin contents can be estimated with digital analysis of color photographs of irises [16]. In future studies, Other factors must also be considered related to variation in prostaglandin-induced iridal hyperpigmentation like the original color of the eye, tyrosinase hyperactivity, melanocyte hyperproliferation, decrease in melanosomes breakdown, and melanocytes response to nerve stimuli [17].

4.3. Quantitative estimation of total melanin contents

Total melanin contents of isolated irides were quantitatively evaluated to make any difference in mean melanin contents of both eyes in all groups. At the dose of 0.5% of inhibitor (Pink group), no difference was observed (right vs left eyes) in the mean melanin contents (μg/g of sample) but in red and blue groups where 1% and 2% inhibitor was used respectively, the difference in mean melanin contents (right vs left eyes) was noted. It means by the increase in doses of inhibitor, the inhibition of latentoprost-induced pigmentation was started and at the dose of 2% of inhibitor (Blue group), significant inhibition was observed (Right eye = 09.560 μg/g (±0.750), Left eye = 13.730 μg/g (±1.062)).

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**Table 2 (continued)**

| Group | Check Points | Right Eye | Left Eye | Right Eye | Left Eye | Right Eye | Left Eye |
|-------|--------------|-----------|----------|-----------|----------|-----------|----------|
|       |              | After 2 months | After 4 months | After 6 months |
| Iris  |              | -          | -        | -         |          | -         |          |
| Pupil Size | 6 mm | 6 mm | 7 mm | 6 mm | 7 mm | 6 mm | 6 mm |
| IOP  | 14          | 17         | 16       | 13        | 17       | 14       | 14       |

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**Table 3(a). Inhibitory effects of α-methyl-DL-tyrosine on latanoprost-induced iridal hyperpigmentation in rabbits by evaluating photographs of the same eye taken at 0 and 6th months by a panel of 5 observers. Data are presented as number (percentage) of observations within each group.**

| Groups                   | Drugs Used                     | No Change in Pigmentation (0 vs 6th Month) | Pigmentation Increased (at 6th Month) | Pigmentation Decreased (at 6th Month) |
|--------------------------|--------------------------------|-------------------------------------------|--------------------------------------|--------------------------------------|
| Pink Group Left Eyes (n = 3) | Latanoprost                  | 5 (33.33%)                                | 10 (66.67%)                          | 0.00%                                |
| Pink Group Right Eyes (n = 3) | Latanoprost + Inhibitor 0.5% | 8 (53.33%)                                | 4 (26.67%)                           | 3 (20.00%)                           |
| Red Group Left Eyes (n = 3) | Latanoprost                   | 4 (26.67%)                                | 10 (66.67%)                          | 1 (6.67%)                            |
| Red Group Right Eyes (n = 3) | Latanoprost + Inhibitor 1%    | 6 (40.00%)                                | 5 (33.33%)                           | 4 (26.67%)                           |
| Blue Group Left Eyes (n = 3) | Latanoprost                   | 3 (20.00%)                                | 12 (80.00%)                          | 0.00%                                |
| Blue Group Right Eyes (n = 3) | Latanoprost + Inhibitor 2%    | 4 (26.67%)                                | 0.00%                                | 11 (73.33%)                          |
| Green Group Left Eyes (n = 3) | Inhibitor 0.5%                | 9 (60.00%)                                | 6 (40.00%)                           | 0.00%                                |
| Green Group Right Eyes (n = 3) | Inhibitor 2%                  | 5 (33.33%)                                | 0.00 %                               | 10 (66.67%)                          |
According to various histopathological, in vitro, and microscopic studies, it has been established that increased iridal pigmentation is due to increased melanin contents in stromal melanocytes instead of an increased number of melanocytes and it is because of increased tyrosinase activity [18]. Tyrosinase is a copper-dependent enzyme located in the membranes of melanocytes. It converts L-Tyrosine into L-DOPA which ultimately leads to the synthesis of melanin and it is the only rate-limiting step in melanin synthesis [19]. This was the rationale and basis behind the use of α-methyl-p-tyrosine as an inhibitor in our current study. The α-methyl-p-tyrosine is being clinically utilized as the drug of choice for the treatment of phaeochromocytoma to block sympathetic activation by inhibiting the tyrosinase enzyme [20]. An in-vitro study conducted on cultured uveal melanocytes obtained from irides revealed that, by the addition of α-methyl-p-tyrosine, latanoprost-induced melanin production was completely prevented [21]. Our results were consistent with this in-vitro study and α-methyl-p-tyrosine belief to be inhibited latanoprost-induced hyperpigmentation by following the same mechanism. In our current study, we calculated total melanin contents although several scientists also worked on the iridal chromophore and concluded that, iris color is due to two distinct macromolecules known as Eumelanin (Brown-black) and Pheomelanin (Yellow-reddish). Eumelanin was a predominantly proven major content in the human iris [22]. This was the limitation of our study, we did not work to differentiate and calculate the macromolecules.

However, concomitant use of latanoprost (Pigmentation inducer) and α-methyl-DL-tyrosine (melanin inhibitor) may be logical to treat

![Figure 2. Heterochromia between the photographs of rabbit's irides taken at 0 months (base line) and 6 months treated with latanoprost only (Left eyes) and latanoprost with different doses of α-methyl-DL-tyrosine (Right eyes) of pink, red, and blue groups. The green group was treated with only α-methyl-DL-tyrosine.](image)

| Group               | No Difference | Pigmentation Increased (Left Eye) | Pigmentation Increased (Right Eye) |
|---------------------|---------------|----------------------------------|-----------------------------------|
| **Pink Group Left/Right (n = 3)** | 7 (46.67%) | 6 (40.00%) | 2 (13.33%) |
| **Red Group Left/Right (n = 3)** | 7 (46.67%) | 7 (46.67%) | 1 (6.67%) |
| **Blue Group Left/Right (n = 3)** | 1 (6.67%) | 14 (93.33%) | 0.00 % |
| **Green Group Left/Right (n = 3)** | 10 (66.67%) | 5 (33.33%) | 0.00% |

Table 3(b). Inhibitory effects of α-methyl-DL-tyrosine on latanoprost-induced iridal hyperpigmentation in rabbits by evaluating photographs of left and right eyes taken at 6th month by a panel of 5 observers. Data are presented as number (percentage) of observations within each group.
glaucoma without the incidence of iridal hyperpigmentation but Longer duration in-vivo and safety studies must be carried out to ensure the production of innovative pharmaceutical products in the future. Besides inhibiting pigmentation, another important and interesting aspect is mentioned frequently in previous literature about the beneficial effects of α-methyl-DL-tyrosine in reducing intraocular pressure in glaucoma patients, but there is no authentic study available to support this claim [23].

4.4. Histopathological evaluation of irides

Since the discovery of latanoprost, its effective use to treat glaucoma and iridal hyperpigmentation as an established side effect, it gained the great interest of scientists to answer the question; of whether this hyperpigmentation is harmful or just a cosmetic effect in unilaterally treated eyes? In this context, several studies have been conducted to evaluate the extent, severity, mechanism, and inhibition of hyperpigmentation as well as histopathological features of latanoprost-treated irides.

In the current in vivo study, besides other studies discussed above, we also checked any histological changes that occurred in irides treated with latanoprost alone or in combination with an inhibitor (α-methyl-DL-tyrosine). Although we could not find any references during the computerized search for similar previous studies like in-vivo inhibition of latanoprost-induced iridal hyperpigmentation and histopathological changes. However, our results were consistent with histopathological studies of iris specimens treated with latanoprost alone and found no evidence of malignant changes in stromal blood vessels and atypical melanocytes as well as hemorrhage of stromal or anterior layers in studied specimens [24]. There was a moderate degree of anterior border layer Pigmentation in 66.7% and 33.3% of specimens in red and blue groups respectively in left eyes whereas in right eyes it was of mild degree. The same findings were also found in several stromal-pigmented melanocytes and Stromal cell pigmentation in red and blue groups. It means α-methyl-DL-tyrosine inhibited pigmentation in the right eyes without any histological changes to the irides. A latanoprost safety study conducted to investigate irides for any malignant changes concluded that there was an increase in melanin contents within the iridal stromal melanocytes without increasing the number of melanocytes and no harmful histopathological findings in latanoprost-treated irides as compared to controls [18]. A molecular biological study of the iris indicates that this activity of melanocytes is majorly dependent upon adjacent cells and their receptors. Fibroblasts are the main neighboring cells, with which melanocytes interact. The prostaglandin in the iris interacts with the FP receptors which are less in melanocytes as compared to fibroblasts, so

Figure 3. Heterochromia between the photographs of rabbit’s irides taken at 6 months treated with latanoprost only (Left eyes) and latanoprost with different doses of α-methyl-DL-tyrosine (Right eyes) of pink, red, and blue groups. The green group was treated with only α-methyl-DL-tyrosine.
their interaction with latanoprost depends upon the FP receptors present on nearby fibroblasts to elucidate signaling pathways at the molecular level [25]. Our above-mentioned other studies were also relevant to the results that at the dose of 1 and 2% of α-methyl-DL-tyrosine, inhibition of pigmentation was noted in the right eye. In the case of free melanin pigments, there was no difference in the left and right eyes of all groups (mild degree in 100% specimens) except the green group (33.3% moderate degree) where only an inhibitor was used. In latanoprost-treated melanin pigmentation was found at a slight tendency in the latanoprost-treated group as compared to the control [26]. It is a matter of interest that the inhibitor alone showed a different response in the green group as compared to other groups concomitantly treated with latanoprost (Pink, Red, and Blue).

Since we used rabbits as an animal model to perform our study and got almost the same results in terms of iris color darkening as Zhan et al performed by using Dutch-Belted rabbits as an experimental model and demonstrated the mechanism of hyperpigmentation. The authors mentioned that sympathetic innervation was involved in iris color darkening and latanoprost may be assumed to play the same role. A similar study was also performed on cynomolgus monkeys to evaluate the latanoprost-induced iridal hyperpigmentation and increased pigmentation was found in sympathectomized eyes. It was further explained in in-vitro studies of human iris specimens containing cultured melanocytes

### Table 4. Mean melanin contents in isolated specimens of irides of rabbits treated with latanoprost (Drug) or α-methyl-DL-tyrosine (Inhibitor) or both. The melanin content is expressed as g/gm of sample (percentage).

| Groups | Latanoprost 0.001% dose | α-methyl-DL-tyrosine as inhibitor dose | Mean Melanin contents (g/gm of sample) | Mean Percent melanin contents (%) | P value (<0.05) |
|--------|-------------------------|---------------------------------------|--------------------------------------|----------------------------------|----------------|
| Pink Right Eye | 1 drop at night | 0.5% (1 drop) | 10.765 (±0.622) | 0.001 (±0.00) | 0.330 |
| Pink Left Eye | 1 drop at night | Nil (Control) | 10.860 (±0.695) | 0.001 (±0.00) | 0.094 |
| Red Right Eye | 1 drop at night | 1% (1 drop) | 14.894 (±1.210) | 0.001 (±0.00) | 0.022 |
| Red Left Eye | 1 drop at night | Nil (Control) | 16.043 (±1.331) | 0.001 (±0.00) | 0.099 |
| Blue Right Eye | 1 drop at night | 2% (1 drop) | 09.560 (±0.750) | 0.001 (±0.00) | 0.330 |
| Blue Left Eye | 1 drop at night | Nil (Control) | 13.730 (±1.062) | 0.003 (±0.00) | 0.00 |
| Green Right Eye | Nil | 2% (1 drop) | 09.932 (±1.817) | 0.001 (±0.00) | 0.099 |
| Green Left Eye | Nil | 0.5% (1 drop) | 10.287 (±0.857) | 0.001 (±0.00) | 0.00 |

### Table 5. Histopathological changes in isolated specimens of irides of rabbits treated with latanoprost (Drug) or α-methyl-DL-tyrosine (Inhibitor) or both in all groups. Data are presented as number (percentage) within each group.

| Histopathology variables | Gradation | Pink Group Right Eye n = 3 | Pink Group Left Eye n = 3 | Red Group Right Eye n = 3 | Red Group Left Eye n = 3 | Blue Group Right Eye n = 3 | Blue Group Left Eye n = 3 | Green Group Right Eye n = 3 | Green Group Left Eye n = 3 |
|--------------------------|-----------|-----------------------------|---------------------------|--------------------------|---------------------------|--------------------------|---------------------------|---------------------------|---------------------------|
| Degree of anterior border layer pigmentation | Mild | 2 (66.7%) | 3 (100%) | 2 (66.7%) | 1 (33.3%) | 3 (100%) | 2 (66.7%) | 2 (66.7%) | 2 (66.7%) |
| | Moderate | 1 (33.3%) | 0 | 1 (33.3%) | 0 | 1 (33.3%) | 1 (33.3%) | 1 (33.3%) | 1 (33.3%) |
| | Dense | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NO: of stromal pigmented melanocytes (To be expressed as cells/HPF) | None | 1 (33.3%) | 0 | 0 | 0 | 1 (33.3%) | 0 | 2 (66.7%) | 2 (66.7%) |
| | Mild | 2 (66.7%) | 3 (100%) | 3 (100%) | 2 (66.7%) | 2 (66.7%) | 3 (100%) | 1 (33.3%) | 1 (33.3%) |
| | Moderate | 1 (33.3%) | 0 | 0 | 1 (33.3%) | 0 | 0 | 0 | 0 |
| | High | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Stromal cell pigmentation | None | 1 (33.3%) | 1 (33.3%) | 3 (100%) | 0 | 2 (66.7%) | 0 | 2 (66.7%) | 2 (66.7%) |
| | Mild | 2 (66.7%) | 2 (66.7%) | 0 | 2 (66.7%) | 1 (33.3%) | 3 (100%) | 0 | 0 |
| | Moderate | 0 | 0 | 0 | 1 (33.3%) | 0 | 0 | 1 (33.3%) | 1 (33.3%) |
| | Heavy | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Stromal free melanin pigment | Mild | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 2 (66.7%) | 2 (66.7%) | 2 (66.7%) |
| | Moderate | 0 | 0 | 0 | 0 | 0 | 1 (33.3%) | 1 (33.3%) | 1 (33.3%) |
| | Heavy | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Stromal inflammation level | None | 2 (66.7%) | 2 (66.7%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) |
| | Mild | 1 (33.3%) | 1 (33.3%) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Moderate | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Dense | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Malignant changes in stromal blood vessels | Present | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Not Present | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) |
| Hemmorhage (Stroma or anterior layer) | Present | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Not Present | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) |
| Stromal mitosis (H&E, without K167) | Not Identified | Not Identified | Not Identified | Not Identified | Not Identified | Not Identified | Not Identified | Not Identified | Not Identified |
| Atypical melanocytes | Present | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Not Present | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) |
treated with latanoprost and iris darkening was induced by tyrosinase expression [27]. We used α-methyl-DL-tyrosine as an inhibitor to block the tyrosinase expression in the synthesis of melanin and it is evident in the outcomes of the right eyes of pink, red and blue groups where α-methyl-DL-tyrosine was used concomitantly with latanoprost.

The strength of our study is that it is the first study of its type (in-vivo) in the world, especially in Pakistan. Although the less number of rabbits per group, shorter duration, and lack of safety studies are the main limitations. It will open new doors for researchers to produce innovative products for the treatment of glaucoma without any concern about hyperpigmentation.

5. Conclusion

These results demonstrate that α-methyl-DL-tyrosine inhibited latanoprost-induced iridal pigmentation in rabbits without causing any harm to the eyes. This effect was started at the dose of 1% but the maximum effect was noted at 2% as a significant difference was noted in the mean melanin contents in the blue group. There were no histopathological changes noted except an increase in pigmentation in the latanoprost-treated eyes.

Declarations

Author contribution statement

Muhammad Sadiq: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Waqar Ahmad: Conceived and designed the experiments.

Muhammad Bilal: Analyzed and interpreted the data; Wrote the paper.

Mahmood Ali: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Mir Azam Khan: Conceived and designed the experiments; Wrote the paper.

Farah Akhtar: Contributed reagents, materials, analysis tools or data.

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Figure 4. Histopathological features of rabbit’s irides specimens taken after 6 months treated with latanoprost only (Left eyes) and latanoprost with different doses of α-methyl-DL-tyrosine (Right eyes) of pink, red, and blue groups. The green group was treated with only α-methyl-DL-tyrosine.
Data availability statement

Data will be made available on request.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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