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Diurnal and Nocturnal Regulation of Intraocular Pressure and Aqueous Humor Outflow Facility in Mice

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Abstract

Intraocular pressure (IOP) is known to fluctuate according to a circadian rhythm. While this phenomenon has been quite extensively studied and reported in a number of species, including mice, to date, there have been few studies on the circadian effect upon total aqueous humor outflow facility (Ctot). In this study, we measured IOP in living anesthetized C57BL/6J mice every 3h over a 24h period, using a TonoLab impact tonometer. We also measured daytime (10:00 pm to 12:00 pm) and nighttime (02:00am to 04:00am) Ctot in anesthetized living mice using a constant flow infusion method. We report that over a 24h period, IOP is highest at midnight (18.34 ± 2.79 mmHg, mean ± St Dev mean) and lowest at midday (12.71 ± 1.42 mmHg), ΔIOP = 5.63 mmHg or 44.3% increase at night, P = 0.005. We also report that Ctot at nighttime (13.66 ± 1.12 nL/min/mmHg ± SEM) is lower than corresponding measurements made during the day time (24.58 ± 2.42 nL/min/mmHg ± SEM), ΔCtot = 10.92 nL/min/mmHg, or 44.4% reduction at night, P = 0.004. In living C57BL/6J mice, IOP and Ctot exhibit circadian variation and appear to be inversely related.

Keywords: Intraocular pressure; Aqueous humor outflow facility; 24-hour variation; circadian variation; mice

Introduction

The regulation of intraocular pressure (IOP) is a critical factor in maintaining ocular health [1, 2]. Chronic elevation of IOP, above its normal value of 10 to 15 mmHg, to values of 21 mmHg or greater is a major risk factor for the development of glaucoma [3, 4], one of the leading causes of irreversible blindness worldwide [5-7]. A key determinant of IOP is total aqueous humor (AH) outflow resistance, often quoted as its reciprocal value, total AH outflow facility, also known as total AH outflow conductance (Ctot). Ctot measures the ease with which AH exits the anterior chamber of the eye. Most of the AH exits through the trabecular meshwork (TM), and the flow rate of AH through this structure changes as IOP changes. A much smaller proportion of AH exits through the uveoscleral and uveovortex pathways[8, 9]. However, over a physiologically relevant range of IOP, the rate of AH flow through the uveoscleral and uveovortex pathways changes very little in response to changes in IOP (unlike the trabecular pathway). Therefore, the uveoscleral and uveovortex pathways incorporate very little outflow facility. It is thus generally assumed that Ctot approximates to C via the trabecular pathway (Ctrab).

The dynamics of Ctot are influenced by various physiological and environmental factors, including aging [10, 11], hormonal changes [12], genetic factors [13, 14], and circadian rhythms, the intrinsic 24-hour cycles governing various biological processes [15, 16]. While many of these factors have been quite extensively studied, and the relationship between IOP and circadian rhythms has been relatively well documented [17], there have been few investigations of the effect of circadian rhythm on Ctot. Further, the studies reported in the literature have been conducted only in human patients [15, 18, 19]. Mice, however, as nocturnal animals, offer a unique perspective on the circadian regulation of ocular physiology. Their active phase during the night and resting phase during the day provides an opportunity to study the intrinsic mechanisms governing Ctot and how these may differ from diurnal species, including humans. Understanding these mechanisms in mice can provide valuable insights into the general principles of ocular physiology and potentially reveal novel therapeutic targets for glaucoma.

The circadian effect on Ctot has been underexplored due to challenges in studying continuous 24-hour cycles. Thus, most research has focused on daytime experimental studies but has neglected potential nocturnal effects. This study aims to elucidate the effect of day and night cycles on Ctot in mice. By measuring Ctot at various time points during the day and night, we seek to uncover any circadian patterns that may exist within the context of this parameter. This research will contribute to a better understanding of how circadian rhythms influence ocular health and may inform the development of chronotherapeutic strategies for the management of glaucoma.

Materials and Methods

Animal Husbandry

The C57BL/6J animals utilized in this study were male and female (assigned to groups on a randomized manner) aged 6 to 8 months and were housed at the University of North Texas Health Science Center vivarium (UNTHSC) in Fort Worth, TX, USA. The animals were provided with standard chow *ad libitum* and resided in cages with dry bedding. The animals were subjected to a 12-hour light-dark cycle, with lights on at 06:30hrs, and were maintained within a controlled environment featuring temperatures of 21–26 °C and humidity levels of 40–70%. All animal studies were conducted under the guidelines and regulations established by the UNTHSC Institutional Animal Care and Use Committee (IACUC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The number of mice used in each experiment is denoted in the representative figures or figure legends. At the end of each experiment, mice were euthanized using CO2(g) inhalation followed by cervical dislocation.

IOP Measurements

Intraocular pressures (IOPs) were measured while the animals were under the influence of gas anesthesia (isoflurane 2.5%; oxygen 0.8 L/min). A TonoLab impact tonometer (Colonial Medical Supply, Londonderry, NH, USA) was used for IOP measurements as previously described [20]. Seven IOP readings were taken on each eye at each time point, and averaged to obtain a final IOP value. IOP measurements during the light phase were conducted under room light, and the measurements during the dark phase were performed under dim red-light conditions. Measurements were conducted every 3 hours over an entire 24-hour period. Care was taken to align the tonometer tip perpendicularly to the central cornea while obtaining the measurements. To minimize the influence of isoflurane on IOP, all measurements were performed immediately when the lid reflex was lost and within a 5-minute window.

Aqueous Humor Outflow Facility

The outflow facilities in both day and night groups were measured in living animals using a constant-flow infusion technique, as previously described [9]. Briefly, mice were anesthetized using a ketamine/xylazine (100/10 mg/kg) solution and maintained at 37 °C using a heating pad. Topical proparacaine HCl 0.5% eye drops were used for corneal anesthesia. Cannulation of the anterior chambers was performed with 32-gauge steel needles (Steriject (½"), TSK Laboratory, Japan) inserted through the cornea, ensuring no contact with the iris or anterior lens capsule. These needles were connected to a BLPR2 pressure transducer (World Precision Instruments (WPI), Sarasota, FL, USA) within a perfusion system. The system was filled with sterile phosphate buffer solution (PBS), and eyes were infused at flow rates ranging from 40 nL/min to 200 nL/min (in 40 nL/min increments) from an SP101i microdialysis infusion pump (WPI). Pressures were recorded over 15-minute intervals, and the mean stabilized pressure at each flow rate was calculated. Total AH outflow resistance was determined from the slope of a plot correlating mean stabilized pressure as ordinate and flow rate as abscissa. The reciprocal of this value returned a value for Ctot. Saline drops were applied topical ocular to prevent corneal drying during the procedure.

Results

Analysis of IOP measurements taken at 3-hour intervals in C57 mice exposed to a 12-hour light/dark cycle confirmed a circadian variation in IOP. The mean IOP was consistently lower during the light phase and higher during the dark phase. (Figure 1A).

This pattern appeared to be sinusoidal. The peak mean IOP measurement was 18.34 ± 2.79 mmHg observed at 2400hrs and the trough mean IOP measurement was 12.71 ± 1.42 mmHg at 1200hrs (n = 10 eyes). The difference between the peak and trough mean IOP measurement was 5.63 mmHg. Statistical comparison of the mean IOP measurements during each phase showed that the mean IOP at 1200hrs was significantly lower than at all other night measurement times ($P < 0.001$).

Additionally, the mean IOP at 2400hrs was significantly higher than at 0900hrs, 1200hrs, 1500hrs, and 1800hrs ($P < 0.001$). The greatest IOP difference between two consecutive observation time points was between 0600hrs and 0900hrs. The 24-hour pattern of each eye in this group was similar in magnitude and frequency (Figure 1B).

Outflow Facility Day vs Night

Measurements in Ctot during both daytime and nighttime periods in anesthetized living C57BL/6J mice using a constant flow infusion technique, as expected revealed a diurnal variation. Daytime measurements were taken from 10:00 AM to 12:00 PM, while nighttime measurements were recorded from 2:00 AM to 4:00 AM. (Figure 2A).

Our findings revealed a significant reduction in Ctot during nighttime compared to daytime. Specifically, the average Ctot at night was 13.66 ± 1.12 nL/min/mmHg (mean ± SEM), which is substantially lower than the daytime average of 24.58 ± 2.42 nL/min/mmHg (mean ± SEM). This represents a decrease in Ctot of 10.92 nL/min/mmHg or a 44.4% reduction at night ($P < 0.005$). (Figure 2B).

These results demonstrate a clear diurnal pattern in Ctot, with significantly lower values observed during the nighttime. The statistical significance of this reduction indicates a robust and reproducible diurnal variation in aqueous humor dynamics in living C57BL/6J mice.

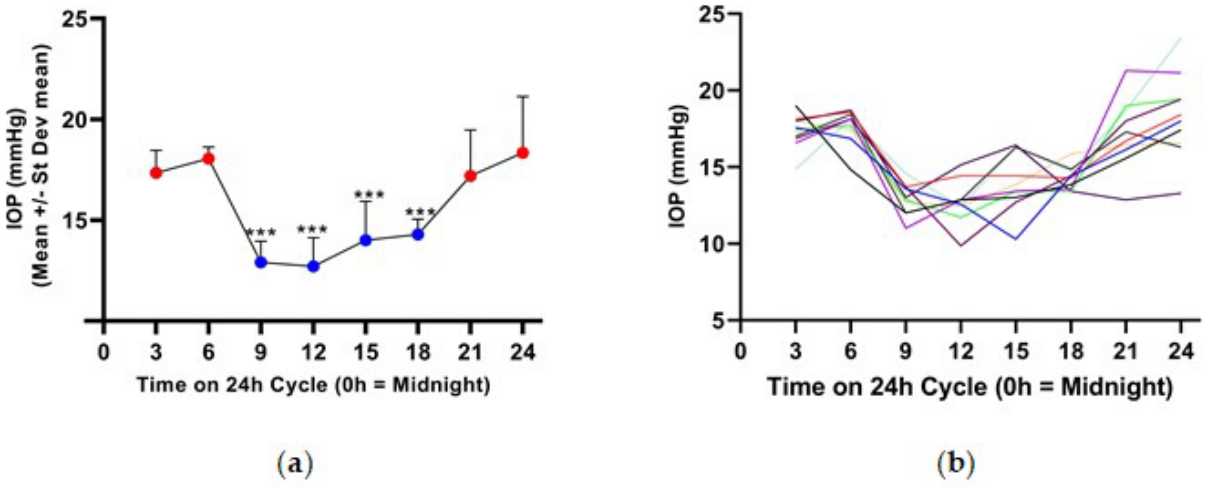

Figure 1: IOP in C57/6J Mice Over a 24h Period (n =10 eyes, 5 animals). (A). Data presented as mean ± St Dev mean. (B) IOP data for each individual eye. Additionally, the mean IOP at 2400hrs was significantly higher than at 0900hrs, 1200hrs, 1500hrs, and 1800hrs ($P < 0.005$). The greatest IOP difference between two consecutive observation time points was between 0600hrs and 0900hrs. The 24-hour pattern of each eye in this group was similar in magnitude and frequency (Figure 1B).

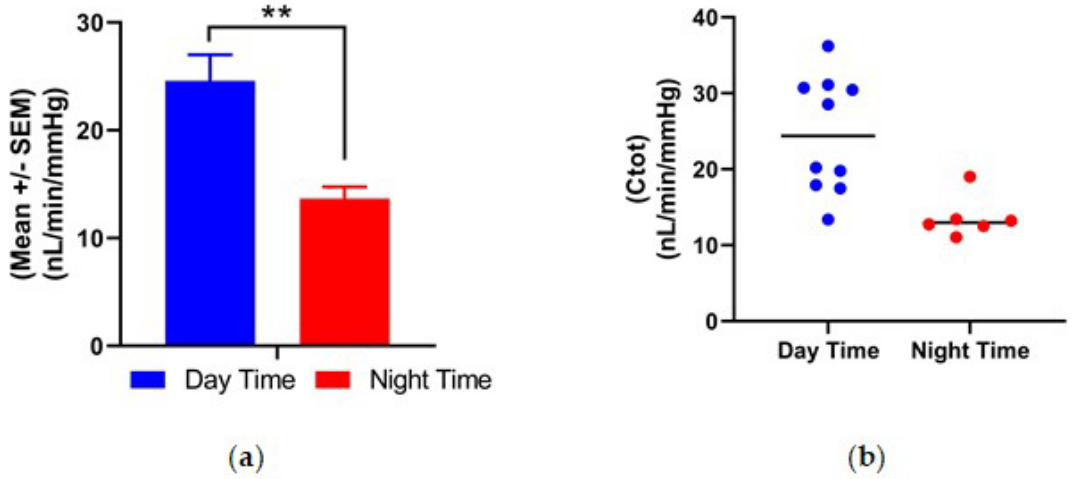

Figure 2: Ctot in C57/6J Mice during both daytime and nighttime periods (n =10 eyes and 6 eyes, respectively). (A). Data presented as mean ± SEM mean. (B) Ctot data for each eye. The average Ctot at night was 13.66 ± 1.12 nL/min/mmHg (mean ± SEM), which is substantially lower than the daytime average of 24.58 ± 2.42 nL/min/mmHg (mean ± SEM). This represents a decrease in Ctot of 10.92 nL/min/mmHg or a 44.4% reduction at night ($P < 0.005$).

Discussion

Circadian rhythms, the intrinsic 24-cycles, have been shown to play a significant role in regulation of IOP. Many studies have reported fluctuations in IOP across the day and night cycle in human and animal models. In both nocturnal rodents and rabbits, as well as diurnal humans, IOP is elevated at night. These fluctuations are thought to be influenced by the circadian regulation of both the rate of AH secretion, and Ctot. AH secretion rate exhibits a diurnal cycle, and has been shown to be significantly reduced at night in humans. There are only a few reports dealing with circadian changes in Ctot over the 24-hour cycle, and all of these studies have been conducted in human patients. Some conclude that nocturnal Ctot is significantly reduced as compared with diurnal Ctot [15, 19] , while others reported that there was a statistically insignificant tendency towards a slight reduction in nocturnal Ctot as compared to diurnal Ctot [18]. It was also reported that Ctot in human subjects measured at various times between (and including) 8:00 AM and 8:00 PM exhibited a significant diurnal variation, within this time-frame [16]. In the present study, we have shown that nocturnal Ctot is reduced in C57/6J mice as compared with diurnal Ctot, by a factor of 44.4%, which is a greater reduction in percentage terms than that reported in human studies.

The anterior segment of the eye of various species (human, non-human primate, cat, rabbit) incorporates both sympathetic and parasympathetic innervation [21-25]. Stimulation of sympathetic nerves leads to an increase in aqueous humor formation rate in the ex-vivo arterially perfused feline eye[26]. It has also been reported that the trabecular meshwork of non-human primates incorporates adrenergic innervation arising from the sympathetic nervous system [22, 27-29].

Further, β-adrenergic agonists have been reported to increase Ctot in these species [30, 31] and in the ex-vivo arterially perfused bovine eye [32]. Also, in isolated human trabecular meshwork, it has been reported that treatment with epinephrine (which acts as an agonist at both the α and β-adrenoceptor) leads to an increase in hydraulic conductivity (i.e. an increase in C) [33, 34]. Studies by Overby et al. on the C57BL/6 and BALB/c mouse, reveal that while the fundamental mechanisms of sympathetic control are maintained, specific responses and receptor distributions can vary. In this species, parasympathetic fibers exist in the ciliary muscle, and cholinergic fibers and terminals have been identified in the trabecular meshwork [35]. There are also sympathetic fibers present in the episcleral vessels running very close to the trabecular meshwork, although they do not appear to be present in the trabecular meshwork itself [35]. The episcleral vessels also appear to possess fibers that release vasoactive intestinal polypeptide and nitric oxide, also substances that can increase Ctot if released into the trabecular meshwork. Thus, sympathetic innervation of the anterior segment of the eye, which influences (amongst other things) the ciliary body and trabecular meshwork, very likely regulates key functions, including aqueous humor secretion rate, Ctot, and IOP across various species, including humans, primates, and rodents.

At night, there may be altered sympathetic influence on the eye, leading to a change in AH outflow resistance, potentially elevating AH secretion rate, and increased IOP. This could be the case for a nocturnal animal. The differences between nocturnal animals and humans regarding sympathetic drive and AH dynamics highlight the need for species-specific approaches in understanding and treating ocular conditions. For humans with POAG, recognizing the nocturnal IOP patterns can lead to better--tailored treatments that consider the timing and type of medication to manage IOP throughout the day and night effectively. For example, treating glaucoma with beta-blockers (e.g., timolol) can reduce AH production, particularly useful during the day when AH production is higher. However, prostaglandin analogs (e.g., latanoprost) increase uveoscleral outflow rate, hence they can be effective throughout the 24-hour cycle.

The current study incorporates several limitations. A small sample size (10 eyes during the diurnal period day time and 6 eyes during the nocturnal period) were studied. Small sample sizes may not sufficiently represent population variabilities, leading to potentially biased or non-generalizable results. Further, only one strain of mouse (C57BL/6J) was utilized, which could mask potential strain-specific differences in AHD or IOP. In addition, study animals were of mixed sex. Male and female mice might

have different baseline levels or responses, which could affect the outcomes of the study. Further, the study cohort animals were within the age range of 6-8 months only. It would be instructive to study single sex cohorts, and of different age groups, as aqueous humor dynamics in mice are age-dependent [36], and may show some sex-dependence also. In addition, IOP measurements were not continuous, but rather were only taken every 3 hours. Ctot was measured only between 10:00 am to 12:00 pm for day time, and 2:00 am to 04:00 am for night time.

Further, species-specific differences impact the precise regulation of IOP and aqueous humor dynamics. For example, Sit et al. (2008) reported that in human subjects there was a statistically insignificant reduction in Ctot during the nocturnal versus the diurnal period, as well as a significant reduction in aqueous humor secretion rate[15], but despite this, there would have to be a nocturnal increase in episcleral venous pressure, or a large decrease in uveoscleral outflow rate (or some combination of both), in order to maintain IOP at its nocturnal level. In support of this, also in human subjects, Nau et al. (2013) reported that nocturnal uveoscleral outflow rate was reduced by 93% compared with diurnal uveoscleral outflow rate [19]. But in the present study, we used C57BL/6J mice of age 6 to 8 months; in this strain, within that age group, only a very low percentage of aqueous humor (~10 to 15%) flows via the uveoscleral pathway [36]. There would not be a great deal of scope for modulation of uveoscleral outflow rate in these animals, between the diurnal and nocturnal period. However, in the present study, we did not attempt to assess uveoscleral outflow rate, nor episcleral venous pressure. However, by Goldmann considerations (along with published figures for aqueous humor dynamics in C57BL/6J mice of the age group studied [36]), the IOP that we measured during the mid-diurnal period fits well with the corresponding measured value of Ctot. But the larger IOP that we measured during the mid-nocturnal period is unlikely to be wholly accounted for by the measured reduction in Ctot that we saw. It is likely that there was also a corresponding increase in episcleral venous pressure, and possibly even a reduction in uveoscleral outflow rate. To address those limitations, future studies should focus on increasing the sample size by utilizing a larger number of eyes and animals which will enhance statistical power. Additionally, testing Ctot in multiple mouse strains, including both sexes separately, and examining various age groups will provide a broader understanding of AHD across different strains, age groups, and sex. Enhancing measurement frequency through continuous or more frequent IOP monitoring will help capture fluctuations that may occur between existing intervals. Finally, broadening the measurement times for Ctot to include more diurnal and nocturnal cycles will offer a more comprehensive understanding of overall aqueous humor dynamics.

In summary, IOP in C57BL6J mice is highest at midnight and lowest at midday. Ctot measured in the early afternoon is correspondingly higher, and lower when measured late at night. Circadian change in IOP is directly related to circadian change in Ctot. Changes are also likely in episcleral venous pressure and possibly the uveoscleral outflow rate.

Statistics

For all experiments, n refers to the number of eyes. GraphPad Prism 9.0 was used to generate graphs and run statistical analyses (GraphPad, San Diego, CA, USA). Significance was determined at a threshold of $P < 0.05$. Data are presented as mean ± St Dev or mean ± SEM, as indicated. Prior to statistical tests, all data was first tested for normality (Shapiro-Wilk test). An unpaired Student's t-test (two-tailed) was utilized for comparisons of facility data between two groups. For multiple comparisons of IOP data, one-way analysis of variance (ANOVA) was utilized.

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