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Effect of general anesthetics on IOP in elevated IOP mouse model

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Abstract

Elevated intraocular pressure (IOP) is the best recognized risk factor for the pathogenesis of glaucoma and the extent of retinal ganglion cell (RGC) degeneration in glaucoma is closely correlated with the extent of IOP elevation. Therefore, accurately and reliably measuring IOP is critical in investigating the mechanism of pressure-induced RGC damage in glaucoma. However, IOP is measured under general anesthesia in most studies using mouse models and many anesthetics affect the IOP measurements in both human and animals. In the present study, we used a noninvasive approach to measure the IOP of mice with normal and elevated IOP. The approach used mice that were awake and mice that were under general anesthesia. Our results demonstrate that not only the behavioral training enables IOP measurement from conscious mice without using a restrainer, it also significantly improves the consistency and reliability of the IOP measurement. In addition, we provide a direct comparison between awake and anesthetized IOP measurements as a function of time after the induction of general anesthesia with several commonly used anesthetic agents. We found that all tested general anesthetics significantly altered the IOP measurements both in normal eyes and in those with elevated IOP. Therefore, we conclude that behavioral training of mice can provide an approach to measure awake IOP that does not require general anesthesia and thus produces reliable and consistent results.

1. Introduction

Glaucoma is the leading cause of blindness in the US. Although many risk factors can influence the pathogenesis of glaucoma, the best recognized and documented risk factor is elevated intraocular pressure (IOP). Elevated IOP has been associated with progressive retinal ganglion cell (RGC) degeneration through several possible mechanisms (Buckingham et al., 2008; Crish et al., 2010; Kuehn et al., 2005; Pease et al., 2000; Tezel and Wax, 2007; Tezel, 2009; Kwon et al. 2009), and the extent of RGC damage in glaucoma is closely correlated with the extent of IOP elevation (Chauhan et al., 2002; Guo et al., 2005; Inman et al., 2006; Levkovitch-Verbin et al., 2002). Therefore, accurately and reliably

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measuring the IOP is critical in investigating the mechanism and determining the prognosis of pressure-induced RGC damage in glaucoma.

Understanding the mechanisms by which elevated IOP causes RGC degeneration is critical for the development of new, logical glaucoma treatments. The use of experimental IOP elevation to produce glaucomatous optic neuropathy in otherwise healthy animals is a common method for studying the pathogenesis of glaucoma. Although many animal models have been used to study the glaucomatous RGC degeneration associated with elevated IOP, mouse models are becoming increasingly popular because they enable researchers to take advantage of genetic manipulation. In vast majority of glaucoma studies using mice, the IOP was measured under general anesthesia. However, accumulating evidence has demonstrated that almost all agents for general anesthesia affect the IOP in both human and animals (Almeida et al., 2004; Artru, 1995; Ausinsch et al., 1975; 1976; 1977; Adams and Barnett, 1966; Badrinath et al., 1986; Bito et al., 1979; Burke and Potter, 1986; Bunch et al., 2008; Camras et al., 2010; Erickson–Lamy et al., 1984; Gelatt et al., 1977; 1982; Hahneberger, 1976; Hahnenberger 1976a; 1976b; Hayreh et al., 1991; Hofmeister et al., 2006; 2008; Jia et al., 2000; Johnson et al., 2008; Komaromy et al., 1998; Krupin et al., 1980; Mirakhur et al., 1990; Mowafi et al., 2003; Murphy, 1985; Nagdeve et al., 2006; Sator-Katzenschlager et al., 2002; Schäfer et al., 2002; Schnell et al., 1996). In addition, many anesthetics have been reported to cause neuronal degeneration in CNS (see Istaphanous and Loepke, 2009; Mellon et al., 2007 for review) or may have neuroprotective properties (see Hudetz and Pagel, 2010; Koerner and Brambrink, 2006; Mortier et al., 2000 for review). Therefore, IOP measurements on awake animals would avoid the effect of anesthetics on the IOP measurement and potential anesthesia-induced confounding of experimental results. A recent study showed that measuring awake IOP in rats that have received behavioral training seems to provide reliable measurements of IOP. However, it was considered difficult to train mice for awake IOP measurement due to the lack of docility (Sappington et al., 2010). Therefore, many recent studies measured IOP from untrained conscious mice with the use of a restrainer (Birke et al., 2010; Cohan et al., 2001; Fan et al., 2010; Harazny et al., 2009; Holcombe et al., 2008; Inoue-Mochita et al., 2009; Nissirios et al., 2007; Porciatti and Nagaraju, 2010; Scholz et al., 2008; Shepard et al., 2010; Wang et al., 2005; 2008). Unfortunately, the IOP measured from untrained and restrained conscious mice tends to vary significantly among laboratories and individual animals (Fan et al., 2010; Iwao et al., 2009; Kipfer-Kauer et al., 2010; Schlamp et al., 2001; Scholz et al., 2008), which could significantly compromise the interpretation of the experimental results.

In this study, we attempted to train mice for awake IOP measurements and measured IOP in awake mice with normal and elevated IOP models to determine whether behavioral training would improve the awake IOP measurements. In addition, we examined the effect of several commonly used general anesthetics on the measurements of IOP in mouse eyes to determine the extent of their effect on mouse eyes with experimentally elevated IOP.

2. Materials and Methods

2.1. Animals

Mice (C57Bl/6) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and NIH guidelines. The use of animals in this study was approved by the Animal Care and Use Committee of the University of Utah.

2.2. Training of mice for IOP measurements awake

Similar to the procedure described previous for rats (Sappington et al., 2010), the behavioral training of the mice for awake IOP measurements was divided into two phases. During the first phase, which lasted one week, each of the experimental animals was held in hands by the experimenter for 15–20 minutes daily and acclimated to tactile contact with each eye through the application of anesthetic drops (0.5% procaine) or saline. During the second stage, which lasted ten days, daily measurements of IOPs were taken from both eyes of each mouse under topical anesthesia.

2.3. Anesthetic agents

Four different anesthetic agents were evaluated in each animal: isoflurane (MWI, Meridian, ID, USA), avertin, and a mixture of ketamine (ketamine HCl, Fort Dodge, Iowa, USA) and xylazine (xylazine HCl, Boehringer Inglheim Vet Inc., St. Joseph, MO, USA). Avertin was prepared by mixing 1.5 g avertin (2-2-2 tribromoethanol, Sigma- Aldrich, St. Louis, MO, USA) with 0.93 ml tert-amyl alcohol (2-methyl-2-butanol, Sigma- Aldrich, St. Louis, MO, USA) and 118.5 ml Dulbecco's Phosphate saline solution (Invitrogen, Grand Island, NY, USA). General anesthesia was performed in the following ways. For isoflurane, each mouse was first placed into an induction chamber, containing a mixture of 20% isoflurane and 80% glycerol. The chamber was then sealed with a transparent cover. The mice were continuously monitored through the cover until recumbent. Then the mice were removed from the induction chamber and placed on a custom-designed heated platform and anesthetized with 2% isoflurane in 95% O_2 and 5% CO_2 (600 ml/minute) controlled by a vaporizer (Vopamatic, A. M. Bickford Inc., New York, NY, USA) through a mouse gas anesthesia head holder with nosecone (David KOPF Instruments, Tujunga, CA, USA). Avertin and the mixture of ketamine and xylazine were administered by intraperitoneal injection at a dose of 315 mg/kg (avertin), 13 mg/kg (xylazine) and 87 mg/kg (ketamine), respectively.

2.4. IOP measurements

A Tonolab® rebound tonometer (Icare LAB tonometer, TonoLab, Colonial Medical Supply, Franconia, NH, USA) was used to measure the IOP of awake and anesthetized mice (Morrison et al., 2009; Ohashi et al., 2008; Pease et al., 2006; 2011; Saeki et al., 2008; Wang et al., 2005). IOP measurements in awake and anesthetized animals were performed in both eyes by the same examiner. All IOP measurements in awake and anesthetized mice were obtained according to the manufacturer's recommended procedures with identical animal handling. For IOP measurements of awake mice, one drop of 0.5% proparacaine hydrochloride was applied to each eye before measurement. To obtain awake IOP measurements from the untrained mice, examiners restrained each mouse by holding the skin between its ears and tail. After training, it is sufficient to place the animals on a bench-top and gently hold the skin between the ears during the measurements. For IOP measurements of anesthetized mice, awake IOPs were first measured under topical anesthesia with one drop of 0.5% proparacaine hydrochloride immediately before induction of general anesthesia. The daily IOP reading of each eye was averaged from 5 trials and each trial was averaged from 6 measurements. To control for the diurnal variation in IOP, all measurements were taken between 9 AM and noon.

2.5. Elevated IOP model

C57BL/6 mice aged 12~20 weeks were used for the elevated IOP model. Mice were first trained for measurement of awake IOP and then the mouse model with elevated IOP was generated by intraocular injection of microbeads following a procedure similar to that used in the studies described previously (Chen et al., 2011; Cone et al., 2010; Pease et al., 2011;

Samsel et al., 2011; Sappington et al., 2010). Briefly, mice were anesthetized with intraperitoneal injection of avertin and local application of 0.5% proparacaine hydrochloride ophthalmic solution (Bausch & Lomb, Tampa, FL, USA) on each eye. Tropicamide ophthalmic solution (0.5%, Bausch & Lomb, Tampa, FL, USA) was used to dilate the pupils of both eyes. A 30G needle was applied from the periphery of the cornea to pierce it without damaging the iris or lens. As much of the aqueous humor as possible was allowed to flow out through this needle. The microbeads or same amount of saline were injected into the anterior chamber using a Hamilton syringe (Hamilton Company, Reno, NV, USA), which was first filled with 1 μl air, and then 2 μl of 15 μm microbead (10×10^6 beads/ml, Molecular Probes, Eugene, OR, USA). The fluid was slowly injected into the anterior chamber over 60 seconds and the empty syringe was left in the anterior chamber for an additional 60 seconds. After injection, 0.5% erythromycin ophthalmic ointment (Bausch & Lomb, Tampa, FL, USA) was applied to each eye. The animals were housed in a standard 12/12-hour cyclic lighting condition after the injection. Twenty-four hours after injection of fluorescent microbeads, IOP was measured daily until it reached a plateau (generally 1 week after fluorescent beads injection). The IOPs were then measured in three consecutive days under general anesthesia.

Data analysis

Off line data analysis of IOP recordings was carried out on a PC computer using the software Microsoft Excel (Microsoft, Bellevue, WA, USA) and Igor (WaveMetrics, Lake Oswego, OR, USA). Statistical analysis was performed using the software StatView (Abacus Concepts, Berkeley, CA, USA).

Results

3.1. Behavioral training of mice significantly reduces the IOP readings

We first determined whether behavioral training would alter the IOP measurements of conscious mice. Mice were initially trained for daily holding and tactile contact for one week. After the initial training, ten daily IOP measurements were taken from both eyes of conscious mice under topical anesthesia. Fig. 1A plots the IOPs as a function of time for each eye. It is evident that both the IOPs of individual eyes and the average IOP of all examined eyes decrease with time, especially during the first 4–6 days, indicating that behavioral training of mice could reduce conscious IOP. Fig 1B shows the distributions of the IOP measurements during the first 3 days (Day 1–3) and last 3 days (Day 8–10) of the second phase behavioral training. Clearly, the IOP measurements during the first 3 days of the behavioral training are distributed in a much wider range and the IOP measurements during the last 3 days of the behavioral training are confined to a very narrow range. Consistently, the variation between IOP measurements also decreases with time during behavioral training. Fig 1C plots the variations of the IOP measurements using the standard deviation (SD) between the 5 trials of IOP measurements of each eye as a function of time. It is clear that the SD was significantly bigger during the first 4 days and rapidly decreased to a more steady level 6–7 days after the beginning of the second phase of training. In summary, both the IOP and SD at the end of the training period (day 10) are significantly lower than that at the beginning (Day 1) of the second phase training. On average, the IOP decreased by 18% (17.5 ± 1.5 mm Hg, mean ± SD, at Day1 and 14.3 ± 0.5 mm Hg at Day 10, $p < 0.0001$, $t = 11.243$, paired t-test, $n = 30$) and the SD decreased by 45% (2 ± 0.6 mm Hg at Day1 and 1.1 ± 0.2 mm Hg at Day 10, $p < 0.0001$, $t = 8.408$, paired t-test, $n = 30$) after the behavioral training (Fig 1D).

3.2. General anesthetics significantly reduce the IOP in normal mice

Previous reports have shown that various anesthetics could alter IOP in humans and animal models. We determined whether the commonly used anesthetics, isoflurane, avertin, and a mixture of ketamine and xylazine, affect the measurement of IOP in normal mice. A group of well-trained mice (15 mice, 30 eyes) were employed for the comparison of the IOP measured awake and under general anesthesia. To test each anesthetic agent, awake IOPs were measured under topical anesthesia immediately before induction of general anesthesia. The first postanesthesia IOP was obtained 3 minutes after the mice lost pain reflexes. The time from the administration of anesthesia to the time animals lose pain reflexes varied, but the subsequent measurements were obtained at 5-minute intervals up to 30 minutes. All mice were tested for the four different anesthetics on three consecutive days. However, on each day, only one anesthetic agent or the mixed agents was tested on each mouse.

Fig 2A, 2B and 2C shows the IOPs of individual eyes as a function of time before and after induction of general anesthesia with isoflurane (Iso), avertin (Ave), and a mixture of ketamine and xylazine (K+Z), respectively. It is evident that all anesthetics reduced the IOP at the end of the test (30 minutes). However, isoflurane and avertin caused only a minimum change in the IOP 3 minutes after the induction of general anesthesia while ketamine and xylazine induced a significant increase of IOP 3 minutes after the induction of anesthesia. This is most obvious from the curves averaged from all measured eyes. Fig 2D shows the average postanesthesia IOPs normalized to the awake IOP as a function of time after the induction of general anesthesia. On average, isoflurane reduced the IOP by 5.5% 3 minutes after induction of anesthesia (14.6 ± 0.5 mm Hg awake and 13.8 ± 1.6 mm Hg after anesthesia). This difference is statistically significant ($p = 0.0122$, $t = 2.673$, paired t-test, $n = 30$). On the other hand, avertin reduced the IOP by only 1.2% 3 minutes after induction of anesthesia (14.3 ± 0.6 mm Hg awake and 14.1 ± 0.8 mm Hg after anesthesia). This difference is not statistically significant ($p = 0.1206$, $t = 1.599$, paired t-test, $n = 30$). In contrast, the mixture of ketamine and xylazine significantly increased the IOP by 51.6% 3 minutes after induction of anesthesia (14 ± 0.4 mm Hg awake and 21.1 ± 1.5 mm Hg after anesthesia, $p < 0.0001$, $t = -25.009$, paired t-test, $n = 30$). However, all three groups of anesthetics reduced the IOP reading by 45.3% (isoflurane), 39.2% (avertin) and 47.6% (ketamine+xylazine) 30 minutes after induction of anesthesia, respectively.

3.3. Agents of general anesthesia significantly reduce the IOP in mice with elevated IOP

We further determined whether these anesthetics have a similar effect on the IOP of mice with elevated IOP. A group of mice (23 mice) were first trained for the awake measurement of IOP and then treated with intraocular injection of microbeads. Eleven mice received microbead injection in the right eye and saline injection in the left eye. Another 12 mice received microbead injection on both eyes. Awake IOP was measured on 3 consecutive days before microbead injection and the average of the measurements for the 3 days was used as the baseline before microbead/saline injection. Beginning the second day after the microbead/saline injection, awake IOP was measured every day or every another day for 2 weeks (14 days). Fig 3A (solid and dash gray lines) shows the IOPs of individual eyes as a function of time before and after microbead injection. It is evident that awake IOP was increased in every eye after microbead injection. Injected eyes were divided into two groups based on the average IOP measured between day 2 and day 7 after microbead injection. The average post-injection IOP 30% higher than the baseline is classified as elevated (solid gray lines), while post-injection IOP less than 30% over the baseline is considered “normal” (dash gray lines). Of the 35 eyes injected with microbead, 22 eyes (63%) of 18 mice have elevated IOP. The black lines in Fig 3A shows the average IOPs as a function of time of the groups of eyes with elevated and “normal” IOP after microbead injection and the eyes with saline injection.

We examined the effect of general anesthesia on the IOP of mice with elevated IOP from day 7 to day 9 after microbead injection. The postanesthesia IOPs were measured under general anesthesia using the same procedure that was used to measure IOPs in mice with normal IOP. The measurements were then compared with those for awake IOP. Similar to the mice with normal IOP, the IOPs of individual mice with elevated IOP decrease with time after induction of general anesthesia with isoflurane and avertin (Fig 3B and 3C), while ketamine+xylazine induce a significant increase of IOP followed by a reduction of the IOP (Fig 3D). Fig 3E shows the average postanesthesia IOPs normalized to the awake IOP as a function of time after the induction of general anesthesia. On average, isoflurane and avertin reduced the IOP by 18.7% (23.4 ± 3.1 mm Hg awake and 19 ± 3.2 mm Hg after anesthesia) and 9.3% (23.2 ± 2.7 mm Hg awake and 21 ± 3.6 mm Hg after anesthesia) at 3 minutes after induction of anesthesia, respectively. The differences are statistically significant ($p < 0.0001$, $t = 11.443$ for isoflurane, and $p = 0.0012$, $t = 3.751$ for avertin, paired t-test, $n = 22$). For the mixture of ketamine and xylazine, the IOP increased by 13% (22.9 ± 2.1 mm Hg awake and 25.9 ± 3.9 mm Hg after anesthesia, $p < 0.0001$, $t = -5.989$, paired t-test, $n = 22$) 3 minutes after induction of anesthesia. Thirty minutes after induction of anesthesia, the IOPs were reduced by 59.8% (isoflurane), 53.9% (avertin) and 63.9% (ketamine+xylazine), respectively.

3.4. The effects of general anesthesia on IOP is stronger in mice with elevated IOP

The data presented above showed that although the IOP is much higher in the IOP elevated mice in comparison with the normal IOP mice before general anesthesia, the IOP of IOP elevated mice at 30 minutes after the induction of anesthesia is much closer to that of the normal IOP mice, indicating that the anesthetics seem to have a stronger effect on the mice with elevated IOP. To further address this possibility, we first determined whether the IOP under general anesthesia is correlated with the IOP before general anesthesia. We did this by examining the relationship between the awake IOPs and the IOPs 30 minutes after induction of general anesthesia of mice with both normal and elevated IOP. Figs 4A, 4B and 4C show the scatter plots of awake IOP and the IOP under general anesthesia of mice with normal and elevated IOP and linear regression analysis. It is clear that the correlation between awake IOP and the IOP under general anesthesia is very poor with all anesthetics, suggesting that the IOP under general anesthesia is not proportional to the IOP from conscious mice.

We then directly compared the effects induced by anesthetics on IOP of mice with normal and elevated IOP by comparing the normalized IOP of mice with normal and elevated IOP. Fig 4D shows that all three groups of anesthetics have a stronger effect on the mice with elevated IOP. On average, the IOPs were reduced by 45.3%, 39.2% and 47.6% in mice with normal awake IOP by isoflurane, avertin and ketamine+xylazine, respectively. In mice with elevated awake IOP, the IOPs were reduced by 59.8%, 53.9% and 63.9%, respectively.

Discussion

4.1. Conclusion

In the present study, we used a noninvasive approach to measure the IOP of mice with normal and elevated IOP. The approach used mice that were awake and mice that were under general anesthesia. This study has three significant contributions to the field. First, we described the significance of behavioral training on the IOP measurement of conscious mice. Our results demonstrated that not only the behavioral training enables IOP measurement from conscious mice without using a restrainer, it also significantly improves the consistency and reliability of the IOP measurement. This is clearly reflected by the reduction of the variation of IOP measurement. A surprising but interesting finding is that behavioral

training reduces the average conscious mouse IOP by 18%. This could significantly affect the data interpretation for glaucoma studies using conscious mice. Second, our study provides a quantitative assessment of how commonly used anesthetics affect IOP in mouse eyes with both normal IOP and ocular hypertension. We found that all tested general anesthetics significantly altered the IOP measurements both in normal eyes and in those with elevated IOP. The time courses of the tested anesthetics on IOP measurement provide useful guidelines to avoid the artifacts due to the use of these anesthetics. Third, we described a time-dependent biphasic effect of ketamine with xylazine on mouse IOP measurement, which has not been reported previously. This finding is likely to reconcile an on-going controversy about the effects of ketamine and xylazine on IOP measurement. Taken together, we concluded that the IOP measured from awake mice after behavioral training seems most accurately and reliably to reflect the actual IOP. However, both avertin and isoflurane appear to be suitable for IOP measurement shortly after the induction of anesthesia in situations where an anesthetic agent is unavoidable.

4.2. Behavioral training of mice significantly reduces the conscious IOP

Elevated IOP has been associated with progressive RGC degeneration in several possible mechanisms. First, elevated IOP could have direct effects on RGC degeneration by blocking axonal transport of RGCs, which reduces retrograde axoplasmic flow of RGCs and cause their death from deprivation of neurotrophic factors. Elevated IOP could also persistently reduce the blood perfusion at the optic nerve head and cause tissue hypoxia and cellular stress and malfunction. In addition, the negative effects associated with the primary damage induced by elevated IOP, such as chronic retinal ischemia, glutamate excitotoxicity, and activated glial cells, could cause secondary degeneration of RGCs over time without elevated IOP (Kuehn et al., 2005; Kwon et al. 2009; Pease et al., 2000; Tezel and Wax, 2007; Tezel, 2009). More recent studies have shown that loss of anterograde axonal transport of RGCs due to elevated IOP preceding the loss of retrograde axoplasmic flow of RGCs and possibly attribute to the initiation of RGC degeneration in glaucoma (Buckingham et al., 2008; Crish et al., 2010). Several studies have shown that the extent of RGC damage in glaucoma is closely correlated with the extent of IOP elevation (Chauhan et al., 2002; Guo et al., 2005; Inman et al., 2006; Levkovitch-Verbin et al., 2002). Therefore, accurately and reliably measuring the IOP is critical in investigating the mechanism and determining the prognosis of pressure-induced RGC damage in glaucoma animal models and clinical practice.

Because almost all agents for general anesthesia affect the IOP in both humans and animals (Almeida et al., 2004; Artru, 1995; Ausinsch et al., 1975; 1976; 1977; Adams and Barnett, 1966; Badrinath et al., 1986; Bito et al., 1979; Burke and Potter, 1986; Bunch et al., 2008; Camras et al., 2010; Erickson–Lamy et al., 1984; Gelatt et al., 1977; 1982; Hahneberger, 1976; Hahnenberger 1976a; 1976b; Hayreh et al., 1991; Hofmeister et al., 2006; 2008; Jia et al., 2000; Johnson et al., 2008; Komaromy et al., 1998; Krupin et al., 1980; Mirakhur et al., 1990; Mowafi et al., 2003; Murphy, 1985; Nagdeve et al., 2006; Sator-Katzenschlager et al., 2002; Schäfer et al., 2002; Schnell et al., 1996) and the repetitive use of anesthetics might cause either neuronal degeneration (Istaphanous and Loepke, 2009; Mellon et al., 2007) or neuroprotection (Hudetz and Pagel, 2010; Koerner and Brambrink, 2006; Mortier et al., 2000) in glaucomatous retina, measuring IOP from conscious animals would avoid the un-wanted effects of anesthetics on the IOP measurement and potential anesthesia-induced confounding of experimental results. Towards this end, measuring conscious IOP in rats after behavioral training seems to provide a reliable measurement of IOP. However, it was considered difficult to train mice for awake IOP measurement due to the lack of docility (Sappington et al., 2010). Therefore, many recent studies measured IOP from untrained conscious mice with the use of a restrainer (Birke et al., 2010; Cohan et al., 2001; Fan et al.,

2010; Harazny et al., 2009; Holcombe et al., 2008; Inoue-Mochita et al., 2009; Nissirios et al., 2007; Porciatti and Nagaraju, 2010; Scholz et al., 2008; Shepard et al., 2010; Wang et al., 2005; 2008). However, the IOP measured from untrained and restrained conscious mice tends to vary significantly among laboratories and individual animals (Fan et al., 2010; Iwao et al., 2009; Kipfer-Kauer et al., 2010; Schlamp et al., 2001; Scholz et al., 2008). The average IOP varied from 8 mm Hg to 28 mm Hg in conscious untrained and restrained C57Bl/6 mice (Kipfer-Kauer et al., 2010; Schlamp et al., 2001) and from 9 mm Hg to 16 mm Hg in untrained and restrained B6 mice (Fan et al., 2010; Scholz et al., 2008). The large variation of IOP measurements was also observed from the same strain of mice measured by the same experimenters in the same studies from untrained but restrained mice (Iwao et al., 2009; Scholz et al., 2008). This large variation could significantly compromise the interpretation of the experimental results. Our current study demonstrates that behavioral training of mice could avoid the use of a restraining devise for awake IOP measurement. Although untrained mice are not well suited for measuring awake IOP without a restrainer, they could become accustomed to the procedure, and after behavioral training the awake IOP measurement can be performed as frequently as necessary with consistent results.

The awake IOP measured at the end of behavioral training is more likely to reflect the “rest” IOP for the following reasons. First, after an initial decline of conscious IOP during the first 4 days of behavioral training, IOP reading reaches a plateau and remains consistent afterward, demonstrating the repeatability of the measurements. In addition, the conscious IOP of trained mice is very close to the IOP measured immediately after the induction of general anesthesia by isoflurane and avertin in the current study. Previous reports of conscious IOP and IOP under general anesthesia also showed similar values (Aihara et al., 2003; Sappington et al., 2010; Wang et al., 2005). These results strongly support the reliability of the conscious IOP measurement in trained mice. Therefore, we conclude that the behavioral training of mice could provide an approach that not only measures conscious IOP with reliable and consistent results but that also avoids the potential anesthesia-induced retinal neuron degeneration due to the repetitive use of anesthetics.

4.3. Anesthetics alter the IOP in a very short time

Numerous reports have shown that various anesthetics could alter IOP in humans (Ausinsch et al., 1975; 1976; 1977; Adams and Barnett, 1966; Badrinath et al., 1986; Mirakhur et al., 1990; Mowafi et al., 2003; Murphy, 1985; Nagdeve et al., 2006; Sator-Katzenschlager et al., 2002; Schäfer et al., 2002), monkeys (Bito et al., 1979; Burke and Potter, 1986; Bunch et al., 2008; Erickson–Lamy et al., 1984; Hahneberger, 1976; Hahnenberger 1976a; Hayreh et al., 1991; Komaromy et al., 1998; Krupin et al., 1980), dogs (Almeida et al., 2004; Artru, 1995; Gelatt et al., 1977; 1982; Hofmeister et al., 2006; 2008), cats (Burke and Potter, 1986; Hahnenberger, 1976b), rabbits (Burke and Potter, 1986; Schnell et al., 1996), rats (Jia et al., 2000), and mice (Camras et al., 2010; Jia et al., 2000; Johnson et al., 2008). Most of the anesthetics have been reported to decrease IOP in human and other animals. In this study, we provided a detailed, direct comparison between awake and anesthetized IOP measurements as a function of time after the induction of general anesthesia in mouse eyes and found that all tested anesthetics induced a time-dependent change of IOP. Consistent with previous reports (Buehner et al., 2010; Jia et al., 2000; Johnson et al., 2008; Mirakhur et al., 1990; Mowafi et al., 2003), our study found that both avertin and isoflurane reduce IOP in mice. However, the reduction in IOP with isoflurane and avertin was relatively slow. The IOP remains relatively stable within 3 minutes after the induction of general anesthesia in mice with normal IOP. On the other hand, both avertin and isoflurane have a faster and more significant effect on mice with elevated IOP. Within 3 minutes, avertin and isoflurane could decrease IOP by approximately 10–20%. These results demonstrated that these anesthetics alter IOP in a very short time period, especially in eyes with elevated IOP.

Therefore, avoiding general anesthesia could minimize concerns that anesthetics may alter the results.

Previous reports on the effects of ketamine on IOP are controversial. Some reports showed that ketamine decreases IOP in human (Ausinsch et al., 1976; Blumberg et al., 2007), monkeys (Bunch et al., 2008; Hahnenberger, 1976a), rats (Jia et al., 2000), and mice (Jia et al., 2000; Johnson et al., 2008). Others show that ketamine increases IOP in human (Bar-Ilan and Pessah, 1986; Corssen and Hoy, 1967; Nagdeve et al., 2006; Yoshikawa and Murai, 1971), dogs (Hofmeister et al., 2006), cats (Hahnenberger, 1976b), and rabbits (Ghaffari and Moghaddassi, 2010; Schutten and Van Horn, 1977). We described a time-dependent biphasic effect of ketamine and xylazine with an initial increase of IOP followed by a decrease of IOP. The increase of IOP is very fast (less than 3 minutes) after the induction of general anesthesia. This is followed by a rapid decline of the IOP until the IOP reaches a significantly lower level 15–20 minutes after the induction of the anesthesia. This result implies that the discrepancy among different reports on the effects of ketamine might reflect a time-dependent change of IOP after the induction of anesthesia.

Because the IOP measured under isoflurane and, especially avertin, has a relatively stable period (within 3 minutes after the induction of anesthesia), their use would appear to be more suitable in situations in which an anesthetic is unavoidable. Although avertin seems to be the most reliable agent, isoflurane is the most convenient agent because it is easy to deliver, the induction is fast, the depth of anesthesia is easy to be controlled, and subjects recover from it quickly. Ketamine with xylazine are the least reliable agents tested in this study because there is no time window in which the IOP is stable and close to the conscious value.

4.4. The conscious IOP is unpredictable from IOP measured under anesthesia

It was suggested that conscious IOP might be predicted from the measurement of IOP under general anesthesia (Jia et al., 2000). Our results showed that there does not seem to be a correlation between the IOP measured at the end of general anesthesia (30 minutes) and the conscious IOP. Although the conscious IOP of the mice with elevated IOP (22.9–23.4 mm Hg) is 8.8–8.9 mm Hg higher than that of mice with normal IOP (14–14.6 mm Hg), the average IOP measured 30 minutes after induction of general anesthesia of mice with elevated conscious IOP (8.3–10.6 mm Hg) is only 1–2 mm Hg higher than that of mice with normal IOP (7.3–8.6 mm Hg). The linear regression analysis also showed a poor linear relationship between IOP measured in mice that were awake and those under general anesthesia for 30 minutes, suggesting that the conscious IOP could not be predicted from IOP measured under anesthesia in mice. In addition, a previous study also showed that IOP measurement under anesthesia produces estimated IOPs that are only marginally accurate and the predictions vary significantly both above and below the IOP measured from conscious animals, strongly suggesting that the use of anesthetics for measuring IOP does not reliably represent the true IOP (Jia et al., 2000) unless the IOP is measured very quickly (less than 3 minutes) after the induction of general anesthesia. Therefore, the IOP measured from trained conscious animals seems most accurately to reflect the actual IOP.

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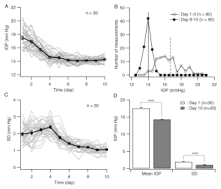

Fig 1. Behavioral training of mice significantly reduces the IOPs

During the second phase of behavioral training, ten daily measurements of IOP were taken from both eyes of 15 conscious mice (n = 30) under topical anesthesia. (**A**) IOP as a function of time of each eye (gray lines) and the average IOP of each day (solid line). The daily IOP measurement of each eye was averaged from 5 trials and each trial was averaged from 6 measurements. (**B**) Distributions of the IOP during the first 3 days (Day 1–3) and the last 3 days (Day 8–10) of the second phase behavioral training. The solid and dash vertical lines indicate the average IOP for the two groups. (**C**) Standard deviation (SD) among the 5 trials of IOP measurements of each eye as a function of time (gray) and the average of the SD of each day (solid line). (**D**) Average IOP and SD at the beginning (Day 1) and the end (day 10) of the second phase of behavioral training. In the panel D of this figure and all following figures, the error bars indicate standard error (SE). * indicates $0.05 > p > 0.01$, ** indicates $0.01 > p > 0.001$, *** indicates $p < 0.0001$. For details of statistical analyses of data presented in all figures, see results section.

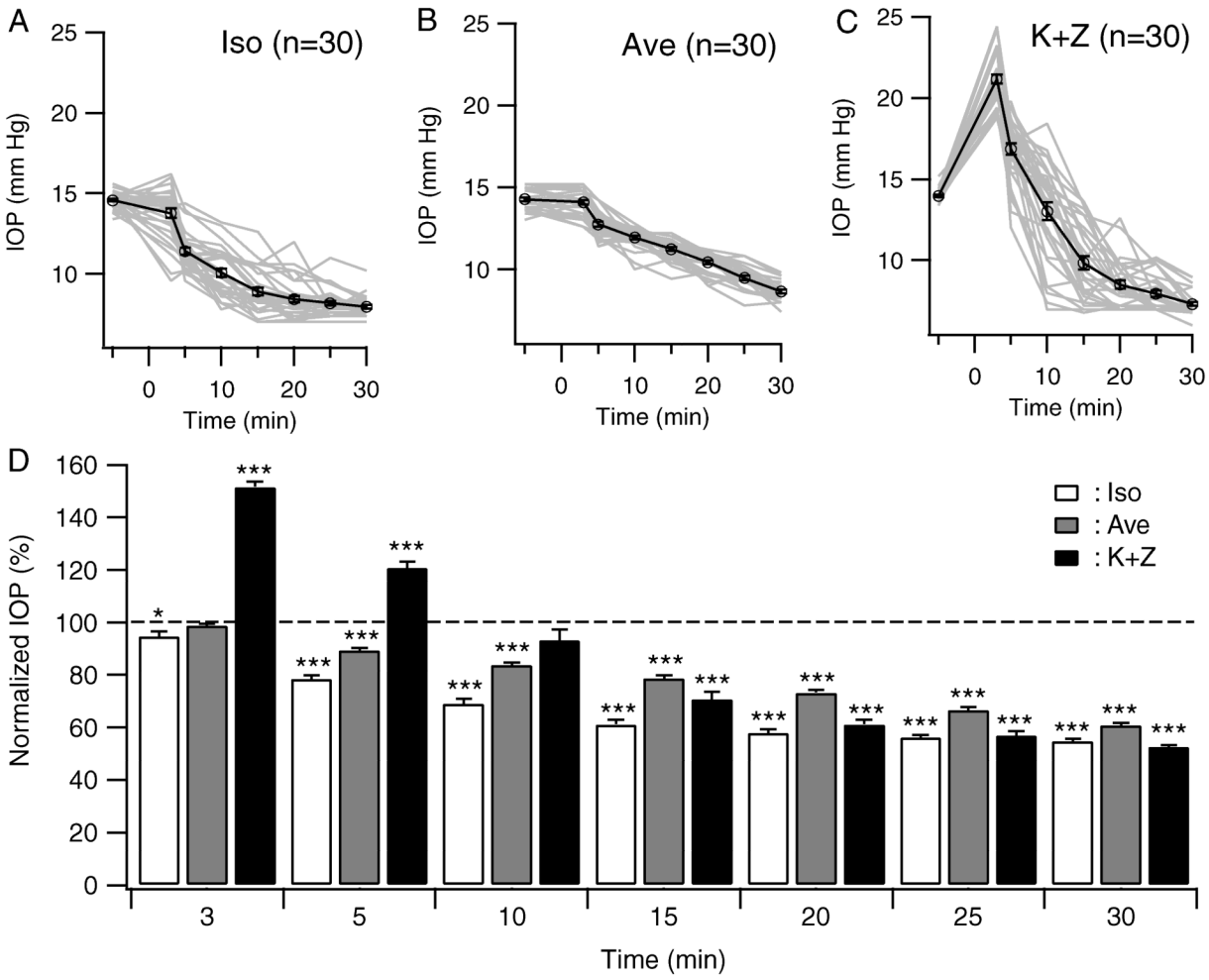

Fig 2. General anesthetics significantly reduce the IOP in normal mice

IOP was measured from both eyes of 15 well-trained mice. Measurements were taken from mice that were awake and mice that were under general anesthesia. (**A**) IOPs immediately before induction of general anesthesia by isoflurane (Iso) and as a function of time after induction of general anesthesia of each eye (gray lines) and the average IOP (solid line). The first point indicates the IOP measured immediately before induction of general anesthesia. The time 0 indicates the time of induction of general anesthesia. (**B**) IOP immediately before induction of general anesthesia by avertin (Ave) and as a function of time after induction of general anesthesia of the same group of mice as shown in panel A. (**C**) IOP immediately before induction of general anesthesia by a mixture of ketamine and xylazine (K+Z) and as a function of time after induction of general anesthesia of the same group of mice as shown in panel A. (**D**) Normalized postanesthesia IOP of mice under general anesthesia by isoflurane (Iso), avertin (Ave), ketamine and xylazine (K+Z) as a function of time after induction of general anesthesia. The horizontal dash-line indicates the conscious IOP measured immediately before induction of general anesthesia.

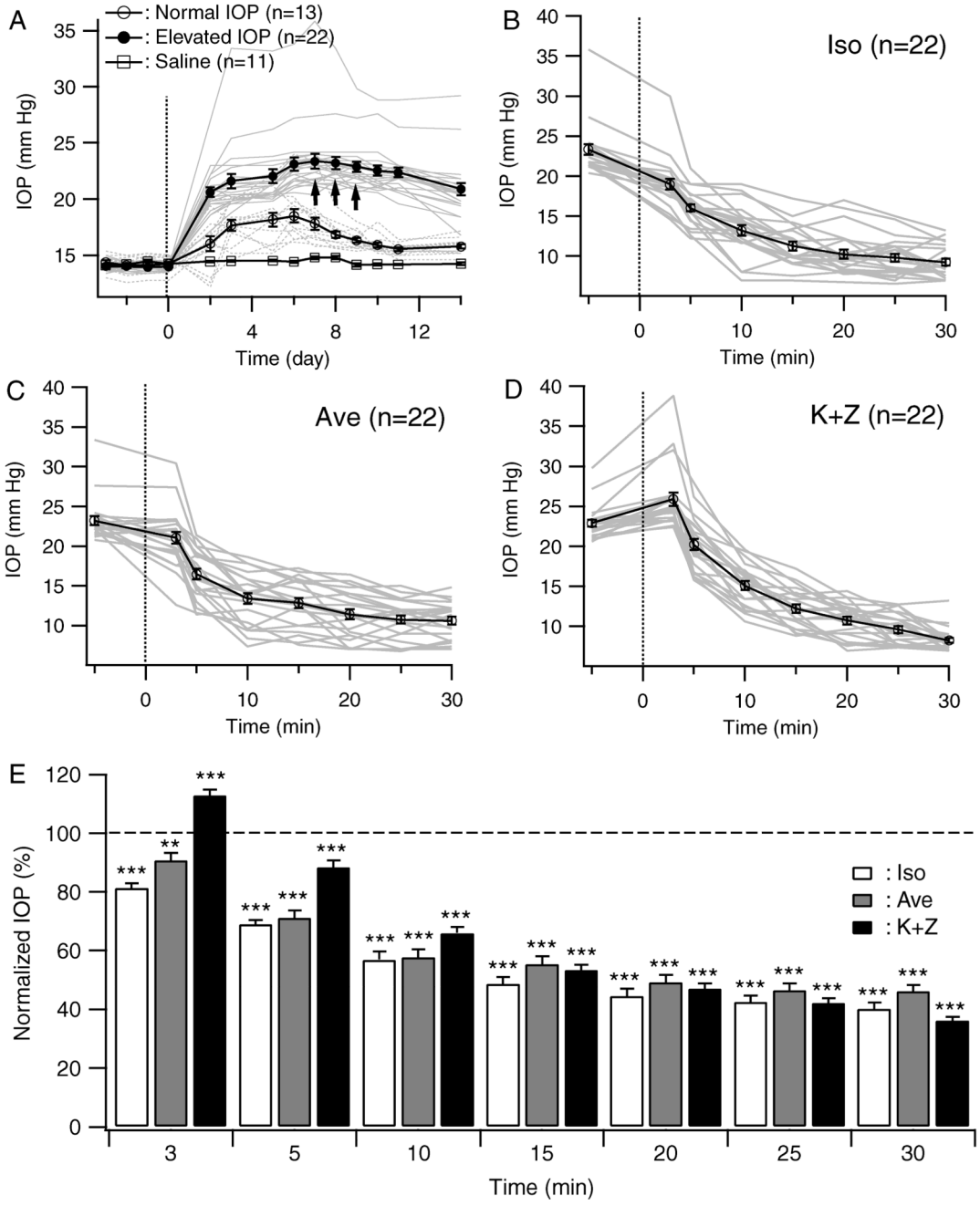

Fig 3. General anesthetics significantly reduce the IOP in mice with elevated IOP

IOPs were measured from 22 microbead injected eyes with elevated IOP, 13 microbead injected eyes with "normal" IOP, and 11 saline injected eyes with normal IOP. Measurements were taken from mice that were awake or mice that were under general anesthesia. The elevated IOP model was generated by intraocular injection of microbeads into 35 eyes of 23 mice. (**A**) IOPs of individual eyes as a function of time before and after microbeads injection (gray lines) and the average IOPs as a function of time before and after microbead or saline injection of the eyes with elevated and "normal" IOP (solid lines). The arrows indicate the days for the tests of general anesthetics. The IOPs of day 0 are averaged from the measurements of 3 consecutive days prior to the microbead/saline injection. (**B**)

IOPs measured immediately before induction of general anesthesia by isoflurane (Iso) and as a function of time after induction of general anesthesia of each eye and the average IOPs (solid line). (**C**) IOPs measured immediately before induction of general anesthesia by avertin (Ave) and as a function of time after induction of general anesthesia of individual eyes (gray lines) and the average IOPs (solid line) of the same group of mice as shown in panel B. (**D**) IOPs measured immediately before induction of general anesthesia by a mixture of ketamine and xylazine (K+Z) and as a function of time after induction of general anesthesia of individual eyes (gray lines) and the average IOPs (solid line) of the same group of mice as shown in panel B. (**E**) Normalized postanesthesia IOPs of mice under general anesthesia by isoflurane (Iso), avertin (Ave), ketamine and xylazine (K+Z) as a function of time after induction of general anesthesia.

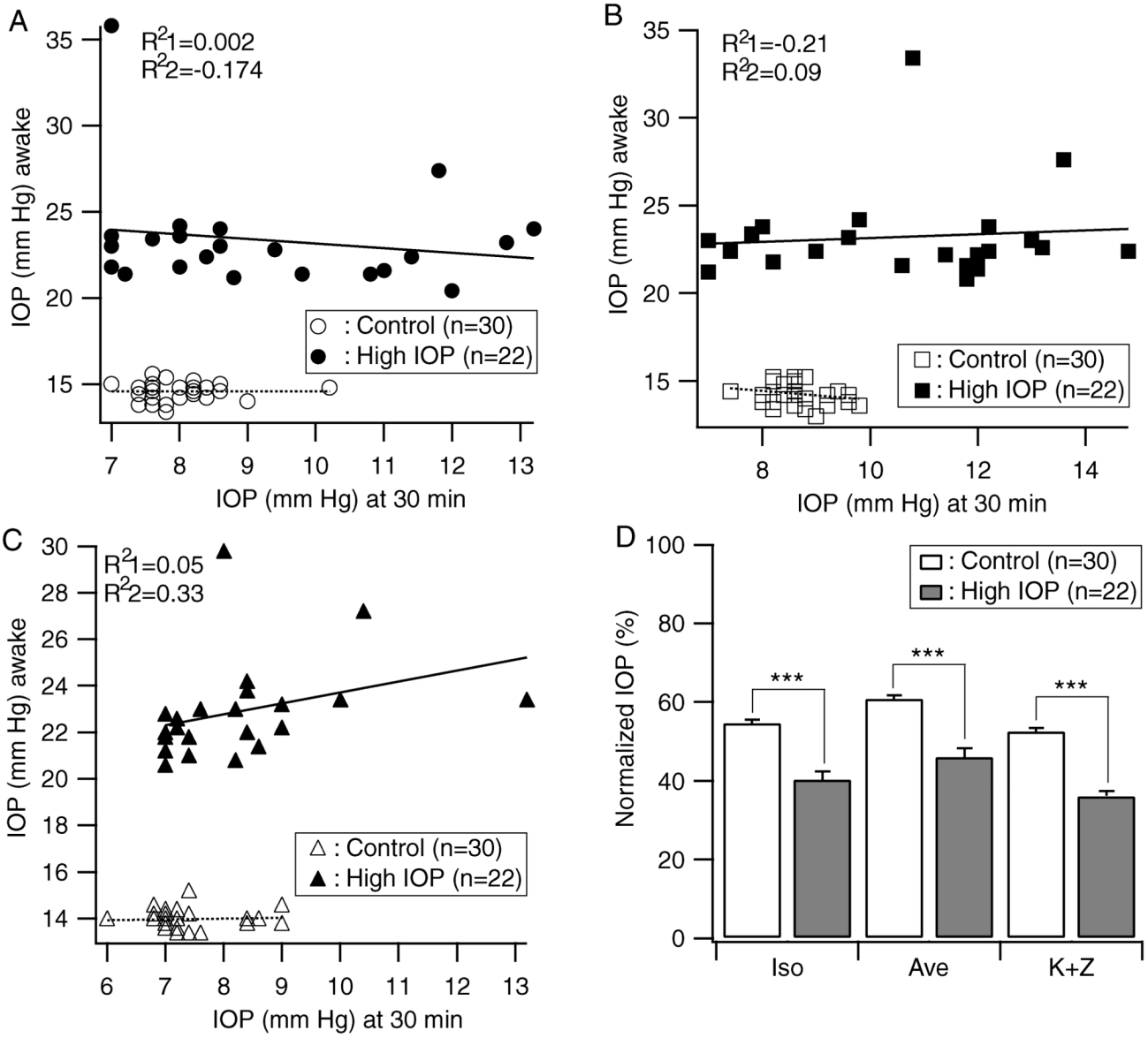

Fig 4. The effects of general anesthetics on IOP is stronger in mice with elevated IOP

IOPs were measured from 22 microbead injected eyes with elevated IOP and 30 un-injected eyes with normal IOP. Measurements were taken from mice that were awake or mice that were under general anesthesia for 30 minutes. (**A**) Scatter plot of the IOPs measured immediately before (IOP awake) and 30 minutes after (IOP at 30 min) induction of general anesthesia by isoflurane (Iso) of mice with un-injected normal (Control) and elevated IOP (High IOP) eyes. Dash-line indicates a linear regression of data from mice with normal IOP. Solid line indicates a linear regression of data from mice with elevated IOP. $R^2 1$ indicates the correlation coefficient of the linear fitting of data from eyes with normal IOP. $R^2 2$ indicates the correlation coefficient of the linear fitting of data from eyes with elevated IOP. (**B**) Scatter plot and linear regression of the IOPs immediately before and 30 minutes after induction of general anesthesia by avertin (Ave) of the same two groups of eyes shown in panel A. (**C**) Scatter plot and linear regression of the IOPs immediately before and 30 minutes after induction of general anesthesia by ketamine and xylazine (K+Z) of the same

two groups of eyes shown in panel A. (**D**) Normalized IOPs of eyes with normal and elevated awake IOP under general anesthesia by isoflurane (Iso), avertin (Ave), ketamine and xylazine (K+Z) for 30 minutes.