

# Reliability of Tonolab measurements in rats

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Received: 2014-01-14 Accepted: 2014-06-20

## Abstract

• **AIM:** To assess the repeatability and reproducibility of Tonolab tonometer in rats with high intraocular pressure (IOP) and evaluate its ability to detect IOP changes in rats with general anaesthesia.

• **METHODS:** Left eyes of adult Fischer rats (F344) were photocoagulated by 532 nm diode laser to induce high IOP. Hypertensive eyes of 30 conscious rats were randomly chosen to measure IOP on a single occasion. Two observers independently and alternately undertook IOP measurements consecutively for three times using the same Tonolab tonometer blind to the other observer's IOP measurements. The within subject standard deviation (Sw), coefficient of variation (CVw) ( $100 \times \text{Sw}/\text{overall mean}$ ), and intraclass correlation coefficient (ICC) were calculated to evaluate intra-observer repeatability. Inter-observer difference was analysed by using 95% limits of agreement described by Bland-Altman and paired sample *t*-test. Also, another 13 normal F344 rats were intraperitoneally administered with ketamine/xylazine or chloral hydrate, and IOPs of both eyes were measured by a single operator once every 5min until animals came to conscious. IOPs at various time points were compared by using one-way ANOVAs.

• **RESULTS:** Mean IOP was 35.58 mm Hg (range 17.33 to 65.33 mm Hg). For intraobserver repeatability, the Sw, CVw and ICC of high IOP for two observers were 5.20 mm Hg/3.41 mm Hg, 9.98%/8.08% and 0.820/0.928 respectively. The inter-observer difference was  $14.76\% \pm 19.76\%$  of the mean IOP of two observers, with a 95% limits of agreement -23.97% to 53.50%, and the difference between mean IOP of these two observers was statistically significant ( $P=0.001$ ). IOPs dropped slightly during the first 15min post-aneesthesia, with a IOP change between 0.17 and 1.17 mm Hg. IOPs changed from baseline of  $11.75 \pm 2.05$  mm Hg ( $n=12$ ) to  $8.75 \pm 1.06$  mm Hg 20min post-anaesthesia ( $P=0.001$ ), and this hypotensive

condition persisted until 80min post-anaesthesia.

• **CONCLUSION:** In this sample of hypertensive rats, Tonolab measurements demonstrated high levels of intraobserver repeatability, however, its interobserver reproducibility was poor. Longitudinal changes of IOP caused by general anaesthesia can be sensitively detected by Tonolab. So we suggested that measurements of IOP using Tonolab are best measured by a single observer, and it could be included in experimental glaucoma.

• **KEYWORDS:** Tonolab; repeatability; reproducibility; rats

DOI:10.3980/j.issn.2222-3959.2014.06.03

Liu LF, Huang CK, Zhang MZ. Reliability of Tonolab measurements in rats. *Int J Ophthalmol* 2014;7(6):930-934

## INTRODUCTION

Rodent models for mimicking glaucoma and evaluating the intraocular pressure (IOP)-lowering mechanism of drugs has been widely used<sup>[1]</sup>. To ascertain the association between IOP and optic nerve damage, accurate and reproducible IOP measurements are indispensable. However, it is difficult to measure IOP in rodents because of their small eyes. Direct measurements with microneedle cannulation into anterior chamber can record IOP directly and accurately<sup>[2]</sup>, but this procedure is invasive and needs to be performed under general anaesthesia, so it cannot be repeatedly used during a short period of time, furthermore, it involves corneal perforation and carries a risk of infection. To overcome these disadvantages, some noninvasive methods of measuring IOP in rodents have been reported<sup>[3,4]</sup>. TonoPen XL electronic tonometer is the most popular one<sup>[5]</sup>. Although there are many reports of IOP measurements using TonoPen in rodents, it was originally designed for use in humans, suggesting it may be less accurate in small animals<sup>[5-8]</sup>.

Since year 2001, the induction-impact rebound tonometer, the first tonometer designed for use in rodents, has become available<sup>[9]</sup>. This is a instrument that is suitable for repeated use, and its lightweight probe makes it possible to measure IOP without anaesthesia. Though the ability of this noninvasive tonometer to reflect true IOP has been reported in several studies, its repeatability, reproducibility and sensitivity to measure IOP in rodents has not frequently been reported, so in this study, we measure IOP in ocular hypertensive and general anaesthetic rats to determine its accuracy and ability to detect changes of IOP<sup>[2,10-14]</sup>.

## MATERIALS AND METHODS

**Animals** Adult Fischer344 (F344) rats (12-16 weeks old) were used in this study (Vital River, Beijing, China). Animals were adapted to an alternating 12h periods of light and dark light schedule (12L:12D, light on at 06:00 a.m.), with free access to food and water. All studies were in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research.

**Study of repeatability and reproducibility** To produce increased IOP we used a diode laser (Vitra, Quantel Medical, France) at 532 nm. Animals were intraperitoneally anaesthetized with a 1:1 mixture (1.5 mL/kg) of ketamine (100 mg/mL), xylazine (20 mg/mL) and topical proparacaine 1% eye drops (Alcaine, Alcon Laboratories, Belgium). Laser treatment was administered unilaterally by 55-70 applications directed towards trabecular meshwork. Laser beam was applied with a power of 440 mW for 0.7s, producing a spot size of 100  $\mu\text{m}$  [15]. One week later, the same procedure were repeated if IOP difference between the treated eye and the fellow eye was less than 8 mm Hg [16]. Topical 5% erythromycin was applied at the end of each procedure.

IOP was measured with TonoLab (Tiolat, Helsinki, Finland) under the manufacturer's instructions in conscious rats. A new disposable probe was used for each animal. Choose the "rat" measurement model. Keep the probe horizontal, start probe-cornea distance about 3-5 mm and angle of 25° limit relative to the visual axis at cornea apex [13]. Only measurements that are judged by data analytical system to be within its parameters are marked as valid: if the measurement is successful, there is a short beep, otherwise, the tonometer will beep twice and show an error message. After effectively measuring for six times, average is calculated and displayed automatically. This machine-generated average is used as IOP for each eye in this study.

Hypertensive eyes of 30 rats (mean IOP 35.58 mm Hg, range 17.33 to 65.33 mm Hg) were randomly chosen to measure IOP with the same Tonolab by two observers in a randomised order, each observer measured for three groups of IOP consecutively. All measurements were completed within 5min, and results were not revealed until all data had been collected. Measurements from a single observer were used to evaluate intra-observer repeatability, and the mean of these three readings was used to assess inter-observer reproducibility.

**Anaesthetic Study** Baseline IOPs of 13 normal F344 rats were measured immediately before induction of anaesthesia. Then, rats were intraperitoneally administrated with 1:1 mixture (1.5 mL/kg) of ketamine (100 mg/mL) and xylazine (20 mg/mL,  $n=6$ ) or chloral hydrate (3 mL/kg,  $n=7$ ). The point when animals reached to a deep anaesthesia (determined by absence of pain and tail-pinch reflex) was defined as

**Table 1** The overall mean ( $\bar{x} \pm s$ ), Sw, ICC and CVw of measurements in ocular hypertensive rats ( $n=30$ )

Observers	Mean (mm Hg)	Sw (95% CI)	ICC (95% CI)	CVw
Observer 1	37.80±3.84	5.20 (2.51-6.91)	0.81 (0.69-0.90)	9.98
Observer 2	33.30±2.61	3.41 (1.74-4.50)	0.93 (0.88-0.96)	8.08

95% CI: 95% confidence interval.

"time 0", and post-anaesthesia measurements were obtained at 5min intervals up to 80min or 60min when animals came to conscious [17]. IOPs in awake and anaesthetized animals were measured in both eyes by the same observer. Care was taken to avoid restraint or eyelid manipulation during all measurements.

**Statistical Analysis** Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA) and Excel (Excel 2003, Microsoft, Redmond, WA, USA).

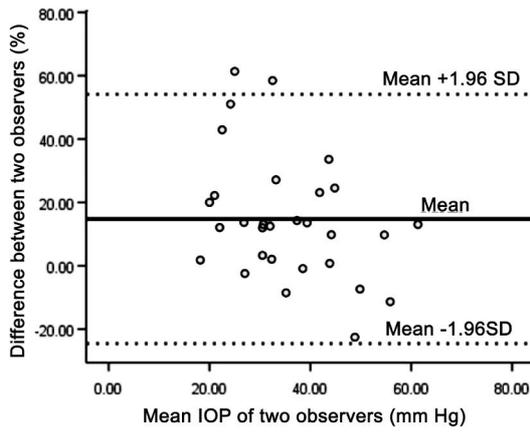
According to formula:  $n=1.96^2/[2 \times 0.2^2 \times (m-1)]$ , where  $n$  represents the number of subjects and  $m$  is the number of observers [18], it was estimated that a minimal sample size would be twenty-five. Within subject standard deviation (Sw) was calculated as the square root of the within-subject mean square of error [19]. Statistical comparison of intraclass correlation coefficient (ICC) was performed by Wilcoxon signed ranks test [20], and ICC values larger than 0.75 may be regarded as acceptable [21]. The coefficient of variation (CVw) were also computed, and a  $CV < 10\%$  was indicative of good repeatability [20]. Bland-Altman method shows the mean difference and 95% limit of agreement between two sets of measurement, and we used this method to evaluate reproducibility of IOP measurements between two observers [22]. The mean IOP of these two observers was also compared by paired sample  $t$ -tests.

In anaesthetic study, average IOPs of each measurement time point in both eyes were determined, and statistical analyses were performed by using one-way ANOVAs followed by Bonferroni's multiple comparison.  $P < 0.05$  was considered statistically significant.

## RESULTS

The mean of high IOP was 35.58±12.00 mm Hg (range 17.33 to 65.33 mm Hg). Intraobserver repeatability was high, with  $CVw < 10\%$ , Sw between 3.41 and 5.20 mm Hg, ICC between 0.81 and 0.93 (Table 1).

Interobserver reproducibility, however, was low. Mean±SD was 4.43±6.49 mm Hg (95% limit of agreement: -8.28 to 17.15 mm Hg). If transformed the bias into percentage, it was 14.76%±19.76% of the mean IOP of two observers, with a 95% limit of agreement -23.97% to 53.50% (Figure 1). Paired sample  $t$ -test showed that the difference between mean IOP of these two observers was statistically significant ( $P=0.001$ ). Awake IOPs in 26 eyes of normal F344 rat were 13.28±2.56 mm Hg (95%CI:12.36-14.20 mm Hg,  $n=13$ ), there was not statistically difference between IOPs of left and right eyes



**Figure 1 Bland–Altman plot of data from the interobserver reproducibility study** Mean IOP for two observers (mm Hg) was plotted against the difference in IOP of these two observers (%). Solid line represents the mean bias and dotted lines represent the 95% limits of agreement. All but two of the data points ( $n=30$ ) lie at or within 1.96 SDs of the mean.

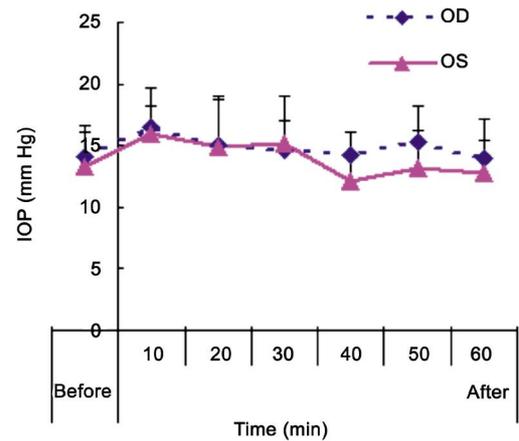
( $P=0.916$ ). For anaesthetics of chloral hydrate ( $n=7$ ), the difference between postanesthetic IOPs (from time point 10min to 1h) and baseline IOPs was not statistically significant in both eyes ( $P=0.072, P=0.676$ ; Figure 2). IOPs between left and right eyes was also not statistically different except for 35min postanesthesia ( $P=0.025$ ). For anaesthetics of ketamine/xylazine, IOP dropped slightly but not statistically during the first 15min post-aneasthesia, with a change between 0.17 and 1.17 mm Hg. However, from 20 to 80min, IOP reduced to a point (7 to 8 mm Hg) that was statistically different from the baseline IOP ( $P=0.000$ ; Figure 3).

**DISCUSSION**

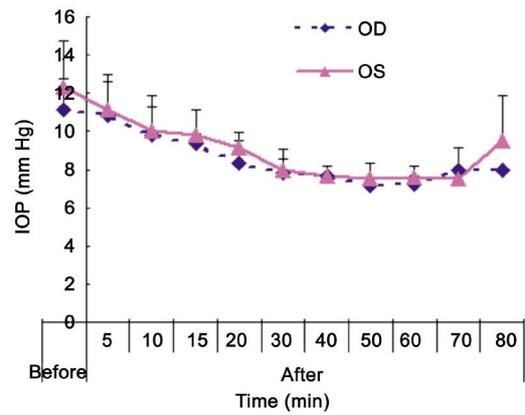
The TonoLab tonometer is designed by the induction-impact mechanism, it pushes a magnetic probe onto the cornea and detects its return-bounce motion with a sensing coil. The motion parameters of the probe vary according to eye pressure and can be used to automatically transform into IOP. Potential advantages of this tonometer include easy portability, longitudinal measurements without anesthesia, and adaptability to small eyes of rodents.

Despite advantages of Tonolab described above, it is necessary to assess its accuracy and sensitivity before usefulness of this tonometer can be confirmed. Repeatability is the variability of the measurements obtained by one person while measuring the same item repeatedly, it represents inherent precision of the equipment. While reproducibility is the variability of the measurement caused by differences in observers' behaviors. So inaccuracy can arise from instruments or systematic differences between observers.

In this study, individual observer appeared to be internally reliable when obtaining repeated measurements (Table 1), indicating that intraobserver repeatability was high. However, when IOPs of two observers were compared, differences in



**Figure 2 IOP dynamics after chloral hydrate anaesthesia in 14 normal rat eyes.**



**Figure 3 IOP dynamics after ketamine/xylazine anaesthesia in 12 normal rat eyes.**

measurement appeared. The average measurement difference ( $4.43 \pm 6.49$  mm Hg) was moderate, with a 95% limits of agreement  $-8.28$  to  $17.15$  mm Hg, if transformed into percentage, this means a  $14.76\% \pm 19.76\%$  bias, with a 95% limit of agreement  $-23.97\%$  to  $53.50\%$ . This large discrepancy could not be accepted in experimental glaucoma. It was also inconsistent with data reported by Lee *et al*<sup>[2]</sup> and Goldblum *et al*<sup>[24]</sup> (Table 2). Lee *et al*<sup>[2]</sup> evaluated the accuracy of the TonoLab in eight cannulated rat eyes by manometrically changing IOP between 20 to 100 mm Hg, and IOPs were measured with a rebound tonometer three times by a single observer. When compared data recorded by Tonolab with the cannulation "golden standard" method by using Bland and Altman method, they got a mean bias of  $7.41\% \pm 7.87\%$ , which was only 50% of ours. Likewise, Goldblum *et al*<sup>[24]</sup> found that the mean difference between induction-impact and cannulation method was  $-3.85 \pm 3.01$  mm Hg, and mean bias was  $\pm 4.6\%$  of mean (range  $\pm 1.9\%$  to  $\pm 7.3\%$ ), which was also much smaller than data in this study. Although we did not include the cannulation method as a comparison in this study because of technical reasons, we can still get our conclusion that the interobserver reproducibility in our study was rather poor. This suggested that although one observer may consistently use the same points for measurement in repeated

**Table 2 Papers about rebound tonometer in rodents searched from PubMed**

Reference	Subjects	Accuracy
Morrison <i>et al</i> <sup>[13]</sup>	Hypertensive Brown Norway rats	Cannulated eyes: R <sup>2</sup> =0.99
Ohashi <i>et al</i> <sup>[5]</sup>	Sprague Dawley rats	R <sup>2</sup> =0.985; circadian change: 2.1±1.1 mm Hg; latanoprost induced change: 3.0±2.1 mm Hg
Lee <i>et al</i> <sup>[2]</sup>	Cannulated rat eyes	R <sup>2</sup> =0.963; mean bias 7.41%±7.87%; limits of agreement -8.02 to 22.84 mm Hg
Pease <i>et al</i> <sup>[14]</sup>	Normal rats; normal mice; hypertensive rats	Normal eyes within 1 mm Hg; glaucomatous eyes: R <sup>2</sup> =0.98; normal mouse eyes: R <sup>2</sup> =0.98
Wang <i>et al</i> <sup>[23]</sup>	Cannulated Wistar rats; different strains of anaesthetic mice	R <sup>2</sup> >0.94; IOP changes: 5.1 to 7.2 mm Hg
Goldblum <i>et al</i> <sup>[24]</sup>	Wistar rats	R <sup>2</sup> =0.6603; mean difference: -3.85±3.01 mm Hg; standard deviations: ±4.6% of mean (range: ±1.9% to ±7.3%)
Kontiola <i>et al</i> <sup>[9]</sup>	Wistar rats	<i>ex vivo</i> R <sup>2</sup> =0.95; <i>in vivo</i> R <sup>2</sup> =0.67

R<sup>2</sup>: Square of the coefficient of multiple correlation, it ranges from 0 to 1, with 0 denoting that model does not explain any variation and 1 denoting that it perfectly explains the observed variation.

**Table 3 IOP history during the first 20min of Ketamine/Xylazine anaesthesia**

Laterality	Conscious	After anaesthesia (min)			
		5	10	15	20
OD	11.17±1.60	10.83±2.14	9.83±1.47	9.33±0.52	8.33±1.21
OS	12.33±2.42	11.17±1.47	10.00±1.90	9.83±1.33	9.17±0.75

**Table 4 IOP history during 60min of chloral hydrate anaesthesia**

Laterality	Conscious	After anaesthesia (min)					
		10	20	30	40	50	60
OD	14.14±2.48	16.43±3.21	15.00±3.70	14.57±2.52	14.29±1.80	15.29±2.87	14.00±3.21
OS	13.29±2.81	16.00±2.16	14.86±4.10	15.14±3.89	12.14±2.41	13.14±3.13	12.71±2.69

examinations, a different observer may choose different points when performing a particular measurement.

To further prove the accuracy of Tonolab, we assessed its ability to detect IOP changes caused by ketamine/xylazine or Chloral Hydrate anaesthesia in normal rats. Our study demonstrated that during the first 15min of ketamine/xylazine anaesthesia, Tonolab could detect a minimal IOP change between 0.17 and 1.17 mm Hg (Table 3). If we analysed all data during the 80min study period (data not shown), the range of IOP change was 0.34 to 4.83 mm Hg. As for chloral hydrate anaesthesia, IOP change was smaller, with range from 0.14 to 2.71 mm Hg (Table 4). This means Tonolab was sensitive enough to detect a minimal change of IOP, which was much smaller than previous reported ones (Table 2). Ohashi *et al*<sup>[5]</sup> evaluated the accuracy of Tonolab by studying nocturnal and Latanoprost induced IOP changes, and found a 2 to 3 mm Hg dynamics. Another study by Wang *et al*<sup>[23]</sup> also reported an anesthesia induced IOP change of 5.1 to 7.2 mm Hg. So, we consider Tonolab could be used in experimental research due to its ability to detect minimal IOP changes. Furthermore, our study also demonstrated that IOP

measured under anesthesia may not reflect true IOP. According to our data, IOP dropped significantly after 15min of ketamine/xylazine anaesthesia. This may due to the mechanism of xylazine, which is a α2-adrenergic receptor agonist, so by directly depressing cardiac function and lowering heart rate, it also has an indirect effect on IOP<sup>[25]</sup>. As for chloral hydrate anaesthesia, IOP did not change significantly, but fluctuation existed. During the first 10min, IOP increased by 2-3 mm Hg, then it tended to decline to the baseline level. This can be explained by the pharmacological properties of chloral hydrate, which plays its role via enhancing the GABA receptor complex and has similar action to barbiturates, so the effect to IOP may be very small<sup>[26]</sup>. To confirm our presumption, we find evidence from other experiments, and result showed that chloral hydrate did not alter IOP levels in both normal and glaucomatous eyes. From all above, we suggest IOP measurement either under ketamine/xylazine or chloral hydrate anaesthesia, the time window should be chosen when IOP is stabilized<sup>[27]</sup>.

In summary, based on our studies, we suggest that before and after measurements of IOP are best measured at the present

time by a single observer, and Tonolab could be included in experimental glaucoma study.

**ACKNOWLEDGEMENTS**

**Foundation:** Supported by National Natural Science Foundation of China (No.30672282).

**Conflicts of Interest:** Liu LF, None; Huang CK, None; Zhang MZ, None.

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