

# Intraocular Pressure Reduction Effect of 0.005% Latanoprost Eye Drops in a Hyaluronic Acid-Chitosan Nanoparticle Drug Delivery System in Albino Rabbits

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**Purpose:** The purpose of this study was to evaluate the intraocular pressure (IOP) reduction efficiency of hyaluronic acid-chitosan-latanoprost link nanoparticle (HA-CS-latanoprost link NP) formulated eye drops.

**Methods:** The IOP reduction study was performed in 24 normotensive albino rabbits. The test animals were randomized and grouped accordingly to treatment namely, HA-CS-latanoprost link NP, plain latanoprost, and the commercially available Xalatan eye drop, all were formulated with 0.005% latanoprost. The 9 days of the experiment were divided into baseline period (days 1–2), treatment period (days 3–6), and recovery period (days 7–9). A wireless noncontact tonometer was used to measure IOP at a time interval of 2 hours for 12 hours per day with 5 readings each.

**Results:** The highest mean daily IOP reduction during the treatment period was 24% for plain latanoprost, 23% for Xalatan, and 29% for HA-CS-latanoprost link NP. The maximum reduction in IOP for plain latanoprost and Xalatan all occurred at the sixth hour with the peak effects of 4.85 mm Hg (37%) and 4.8 mm Hg (36%), respectively. Although HA-CS-latanoprost link NP had peak effects of 5.75 mm Hg (43%) at the sixth hour and 5.22 mm Hg (39%) at the eighth hour. Daily mean IOP measurements of each treatment group showed that HA-CS-latanoprost link NP has a greater IOP reduction effect compared with the other two treatments ( $P < 0.001$ ).

**Conclusions:** The results showed that the formulation of latanoprost with CS and HA is more effective in reducing the IOP than by drug alone.

**Translational Relevance:** The results provide evidence from animal experiment that HA-CS-latanoprost link NP formulation could improve and sustain drug concentration in the anterior segment of the eye. The improved reduction in IOP with that HA-CS-latanoprost link NP formulation can serve as a basis that latanoprost eye drops can be formulated with decreased concentration of benzalkonium HCl, an irritant preservative and penetration enhancer.

## Introduction

Glaucoma is the leading cause of irreversible and treatable blindness worldwide.<sup>1</sup> It leads to optical nerve degeneration and death that is linked to elevated intraocular pressure (IOP). In all types of glaucoma, the increase in IOP is brought about by the decrease in the outflow of aqueous humor (AH) through the trabecular meshwork.<sup>2</sup> Most cases of glaucoma are primary open-angle glaucoma (POAG), which leads to chronically elevated eye pressure, and eventually gradual loss of sight.<sup>3,4</sup> It was estimated that people who will be affected by glaucoma and POAG will increase to almost 112 million and 80 million in 2040, respectively.<sup>5</sup> The optic nerve damage in POAG can occur at a wide range of IOPs, and the rate of progression is highly variable. Although the majority of patients have elevated IOP, at least one-sixth of patients with POAG have IOP levels below 21 mm Hg, which is called normal-tension glaucoma (NTG).<sup>2,6</sup>

Even though elevated IOP is absent in some glaucoma cases, it is still a relevant risk factor for POAG and there is substantial evidence that controlling IOP reduces the progression of glaucoma in approximately 90% of cases, including cases of NTG.<sup>7</sup> To prevent vision impairment and loss due to glaucoma, it requires long-term management with medications that reduce IOP. Moreover, it is important for patients to adhere to their drug regimen to prevent or retard vision loss.<sup>5</sup> The most favorable and dominant route of drug administration to the ocular site is topical instillation because it is the simplest, most convenient, noninvasive procedure, and can be self-administered.<sup>8</sup> However, because the anatomy of the eye is naturally protected from foreign matter, ophthalmic drops are associated with very variable therapeutic efficiency, poor bioavailability (less than 10%), high reliance on patient compliance, and side effects due to chronic use.<sup>9–11</sup>

Currently, prostaglandin (PGF<sub>2α</sub>) analogs are considered the most potent agents to improve uveoscleral outflow and are more effective in decreasing IOP compared with other classes of ocular antihypertensive drugs.<sup>4,12</sup> Latanoprost is the first-line of treatment for POAG and it has the advantage over other PGF<sub>2α</sub> analogs of having less irritation to the ocular surface.<sup>13</sup> Studies have consistently showed that the safety profile of latanoprost is equal to or superior to the other PGF<sub>2α</sub> analogs.<sup>14–17</sup> A study comparing benzalkonium HCl (BAC) free bimatoprost and latanoprost formulation showed that hyperemia scores are significantly lower in latanoprost.<sup>15</sup> An efficacy study review of latanoprost, bimatoprost, travoprost,

and timolol in the management of POAG, stated bimatoprost to be most effective but has the highest risk of hyperemia, whereas latanoprost has the lowest risk.<sup>17</sup> A study in Japanese with NTG, showed that there is no significant difference comparing the IOP reduction efficacy and safety between latanoprost and tafluprost.<sup>14</sup> The efficacy profile of latanoprost is similar to other PGF<sub>2α</sub>, and long-term patient adherence to it is facilitated with its safe and tolerable profile, making it a balanced treatment of POAG.<sup>16</sup> However, latanoprost, like other PGF<sub>2α</sub> analogs, has been associated with side effects, such as blurring of vision, burning, stinging, watering and itching of the eyes, browning of the iris, darkening of the eyelid skin, growing of eyelashes, and reddening of the conjunctiva.<sup>18</sup> Additionally, the design of the formulation and selection of excipients can influence the degree of irritation.<sup>19</sup> The latanoprost ophthalmic solution currently available in the market (Xalatan) contains 0.02% of benzalkonium chloride (BAC), which has four times greater the amount of BAC compared to the usual 0.005%. BAC is used both as a preservative and corneal penetration enhancer.<sup>20</sup> It acts as a penetration enhancer by weakening the tear film, mucous layer, and phospholipids that impedes drug access.<sup>21</sup> BAC is an irritant and results in a variety of side effects in the eye, such as inducing inflammation and drying of the eye, conjunctival allergy, and changing of the corneal epithelium.<sup>4,22</sup> Eye irritation and other adverse effects could be reasons for patient noncompliance to their medication regimen.<sup>19</sup> Poor patient adherence led to poor management of IOP and disease progression with eventual loss of vision.<sup>23</sup> Thus, to improve patient compliance and avoid those side effects that would add up to problems regarding long-term medications for glaucoma, suggested remedies are switching to other eye drop preservatives, reduction in the amount of BAC, or use no preservatives at all.<sup>24</sup> To reduce the use of BAC as a preservative and penetration enhancer, the addition of other penetration enhancers, such as polymeric nanoparticles (NPs) in the eye formulation may be done.

This study evaluated the IOP reduction efficiency of linking latanoprost with biodegradable polymers chitosan (CS) and hyaluronic acid (HA) or hyaluronic acid-chitosan-latanoprost link NP (HA-CS-latanoprost link NP) formulation, for improved ocular drug delivery. It is a NP drug delivery system that may improve ocular bioavailability and address previously mentioned problems with the currently available form of latanoprost. Chitosan (CS) is a biodegradable, nontoxic, mucoadhesive that binds to the negatively charged sialic acid in the mucin layer of the cornea and conjunctiva through hydrogen bonding and

hydrophobic interaction.<sup>25,26</sup> It is a new drug delivery system that may improve ocular bioavailability and address previously mentioned problems with the currently available form of latanoprost.<sup>11,27</sup> It is also characterized by its property to enhance drug penetration by reversibly opening tight junctions between epithelial tissues.<sup>8</sup> Hyaluronic acid (HA) is another naturally occurring mucoadhesive polysaccharide that can enhance cellular targeting by binding to CD44 receptors found in the cornea and conjunctiva.<sup>26</sup> Nanoparticles (NPs) will spontaneously form with the ionotropic gelation (IG) method, where positively charged CS interacts with anionic triphosphate (TPP) under mechanical stirring at room temperature.<sup>28</sup> They form NPs with a functional group surface that allows it to adhere to the mucus layer, thus allowing the drug's drainage to be controlled by the mucus turnover time rather than the tears. The large surface area and mucoadhesiveness of these NPs will allow prolonged release of the drug and increased penetration, thus will improve ocular availability of the drug.<sup>29</sup> The prolonged release of the drug and enhanced penetration could serve as a basis that these mucoadhesive NPs can improve drug concentration to the active site and in a controlled manner; decrease drug side effects by preventing unwanted drug distribution; and a basis for decreasing the amount of BAC as preservative and penetration enhancer or replacing it with a less toxic preservative.

## Methods

### Materials

Hyaluronic acid-chitosan-latanoprost link nanoparticle (HA-CS-latanoprost link NP) eye drops (2020) were prepared using latanoprost, hyaluronic acid, benzalkonium chloride, sodium chloride, sodium dibasic phosphate, sodium monobasic phosphate, and water for injection (WFI) obtained from EL Laboratories, Inc. (Laguna Technopark, Philippines) and sponsored by Vista Pharma, Inc. (Taguig, Philippines); low molecular weight CS (degree of deacetylation: 77.0%) and sodium triphosphate were purchased from Sigma Aldrich (St. Louis, MO, USA).

Plain latanoprost solution has all the materials listed above except for the HA, CS, and sodium triphosphate. Xalatan eye drop formulation was purchased as a commercial product.

The chitosan-latanoprost link NPs (CS-latanoprost link NP) was formed using the ionic gelation method. Cross-linking between the CS polymer chain was brought about by the dropwise addition of TPP. The NPs will spontaneously generate by binding of the positively charged amino groups of CS and the anionic functional groups of the cross-linker TPP under agitation at room temperature.<sup>30,31</sup> Drug molecules may bind in the matrix of the polymer by electrostatic, hydrogen bonding, and hydrophobic interactions.<sup>31</sup> Hyaluronic acid (HA) is a multi-negatively charged polymer, thus upon the formation of CS-link NP the addition of the HA molecules allow it to be adsorbed on to the exterior of positively charged CS-NPs. The description of the composition and characteristics of the HA-CS-latanoprost link NPs are found in [Tables 1 and 2](#).

### Animals

The experimental protocol of this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Santo Tomas Philippines with approval no. RC2018-621018 (October 18, 2018). The submitted and approved protocol was strictly followed and it complies with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Twenty-four (24) albino rabbits (*Oryctolagus cuniculus*) weighing 2.5 to 3.0 kg were obtained from KC Rabbitry, a pioneer rabbit breeder in the Philippines with the Department of Agriculture Certification.

They were housed at the Thomas Aquinas Research Complex Animal House of the University of Santo Tomas with standard environmental conditions, a temperature of 23°C ( $\pm 2^\circ\text{C}$ ), humidity at 40 to 70%, and a light cycle of 12 hours light and 12 hours dark.<sup>20,32</sup> Two rabbits were kept in a metal cage having the size of 26 × 13 × 12 inches and were separated from each other by a divider. Cages, water containers,

**Table 1.** Description of the HA-CS-Latanoprost Link NP Composition

Ingredient	Description (Use)
Latanoprost	Drug substance that reduces IOP pressure
Chitosan	Positively charged mucoadhesive and penetration enhancer polymer
Sodium triphosphate	Anionic cross-linking agent
Hyaluronic acid	Negatively charged substrate for CD44 receptors and mucoadhesive polymer

**Table 2.** Characteristics of HA-CS-Latanoprost Link NPs

Parameters	Evaluation, Mean $\pm$ SD	
	CS-Latanoprost-Link NP	HA-CS-Latanoprost-Link NP
Particle size, nm	199.7 $\pm$ 2.25	314.4 $\pm$ 0.630
Poly dispersibility index	0.274 $\pm$ 0.010	0.431 $\pm$ 0.0121
Zeta potential, mV	34.97 $\pm$ 0.757	29.87 $\pm$ 0.513

\*Data are presented as mean  $\pm$  SD;  $n = 3$ .

and the feeders were disinfected daily with 10% sodium hypochlorite. The animals were fed with Hagen Rabbit Pellets twice daily and vegetables twice a week, and were given a suitable amount of distilled water.

### Intraocular Pressure Reduction Activity

All of the experimental animals provisionally selected for testing were physically examined, especially the eyes, by a licensed veterinarian. It was made sure that the animals showing eye irritation, ocular defects, or pre-existing corneal injury were not included in the experiment. Normotensive albino rabbits weighing 2.5–3.0 kg were used for the IOP reduction study. The animals were acclimatized for at least 7 days before the start of the experiment.

The 24 albino rabbits were divided into 2 batches. The 2 batches, each with 12 rabbits, have undergone the same IOP reduction test, the same acclimatization period, and the same environmental conditions stated above. This was done to allow the measurement of IOP within 12 hours with a 2-hour interval by a single person and to arrive with 7 IOP readings per test animal.

Rabbits in each batch were assigned with numbers 1 to 12 and were randomly grouped having 4 rabbits in each group. Each group was assigned with treatment, namely plain latanoprost, the commercially available Xalatan, and HA-CS-latanoprost link NP formulated eye drops, all having 0.005% latanoprost. The placebo solution is composed of a normal saline solution (NSS).

The 9-day experiment was divided into consecutive periods of baseline (days 1–2), treatment (days 3–6), and recovery (days 7–9). No medication was given during the baseline period. Assigned medications were given during the 4-day treatment period, which was discontinued on days 7 to 9 during the recovery period. Using sterile eye droppers, a drop of the assigned treatment was instilled on the right eye while NSS (a drop) was instilled on the left eye of each rabbit. The medications were given once daily at 8 AM during the treatment period.<sup>32</sup>

Intraocular pressure (IOP) measurement was performed throughout the 9 days of the experiment. During the treatment period (days 3–6), IOP in both eyes of each rabbit was measured immediately before drug instillation, then every 2 hours after the instillation of the treatment solutions for 12 hours. During the baseline and recovery periods, IOP measurement was performed at the same time points as the treatment period. There were five readings done for each time point for both eyes of each rabbit. The IOP measurement was performed by the same person and was blinded to the treatment provided to the rabbits. Restraining boxes were used during the instillation of eye drops and measurement of IOPs. Intraocular pressure (IOP) was measured using the TonoCare handheld, wireless, noncontact tonometer (Keeler Ltd., Windsor, UK), which does not require instillation of anesthesia before use. The formula used for IOP reduction and percentage (%) IOP reduction is as follows:

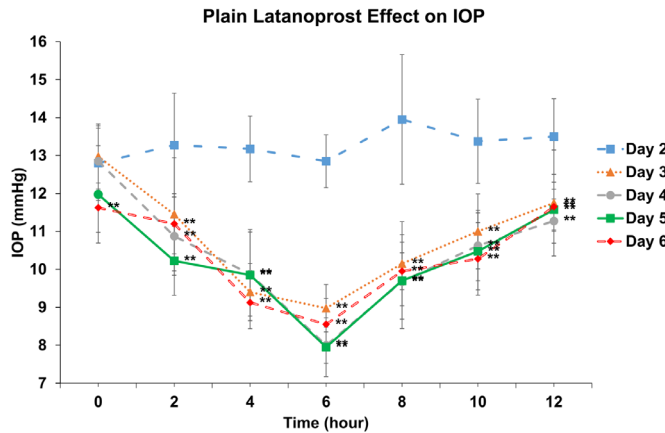
$$\begin{aligned} \text{IOP reduction} &= x - \text{IOP of the 0}^{\text{th}} \text{ hour of Day 2 (control/baseline)} \\ \% \text{ IOP reduction} &= \frac{x - \text{IOP of the 0}^{\text{th}} \text{ hour of Day 2} \times 100}{\text{average IOP measurement in day 2}} \end{aligned}$$

where  $x$  = IOP of the remaining time points of day 2 and treatment days (days 3–6).

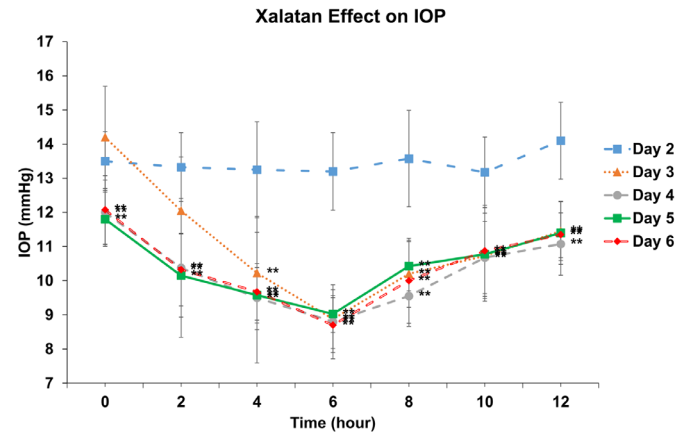
It is based upon the principle of measuring the change in the IOP after instillation of an IOP lowering medication on the eyes of normal albino rabbits.<sup>33</sup>

### Data Analysis Process

The IOP reduction for baseline days 1 and 2 and treatment days 3, 4, 5, and 6 were computed. Day 2 was considered to be the baseline and not day 1 due to unequal variances (tested using Levene's test) and external nuisance factors (measurement error, interaction with rabbits, etc.). Eight data points were gathered per time interval because there were eight rabbits per treatment (2 batches with 4 rabbits). The mean of the eight data points per time interval was computed then plotted to have a graphical representation of the



**Figure 1.** Effect of 0.005% plain latanoprost eye drops on IOP at 0 to 12 hours throughout treatment days 3, 4, 5, and 6 compared with baseline day 2,  $**P < 0.005$ ;  $n = 8$ .



**Figure 2.** Effect of 0.005% Xalatan eye drops on IOP at 0 to 12 hours throughout treatment days 3, 4, 5, and 6 compared with baseline day 2,  $**P < 0.005$ ;  $n = 8$ .

data. Wilcoxon Signed-Rank Test at 5% level of significance was used to determine the difference between IOP reduction at baseline and treatment periods and between treatments per time interval. The significant difference of daily average IOP measurements between treatments was computed using the Friedman test at a 5% level of significance.

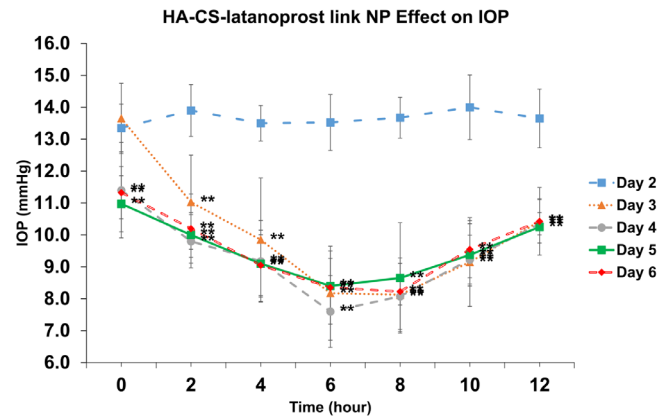
To determine if the IOP would return to normal (have a similar distribution to the first day or baseline), the Friedman test was also conducted on the IOP distribution per treatment on the recovery days 7, 8, and 9 against the IOP distributions on day 1. The Wilcoxon Signed Rank test at 5% level of significance was used to compare the mean IOP measurements of all treatments during recovery days.

## Results

### Effect of HA-CS-Latanoprost Link NP, Plain Latanoprost, and Xalatan on IOP

The comparison between the IOP measurements during treatment days 3, 4, 5, and 6 and baseline day 2 are shown in Figures 1, 2, and 3. At 5% level of significance, the mean IOP measurements at treatment days were significantly lower than the IOP measurements at baseline, 24 out of the 28-time intervals (86%) for the plain latanoprost, 26 out of the 28-time intervals (93%) for the Xalatan, and 27 out of the 28-time intervals (96%) for the HA-CS-latanoprost link NP treated groups. The  $P$  values are presented in Supplementary Tables S1, S2, and S3.

With plain latanoprost eye drops, reduction of IOP started at day 3 wherein at 5% level of significance, IOP reduction was significantly different at the 4th to



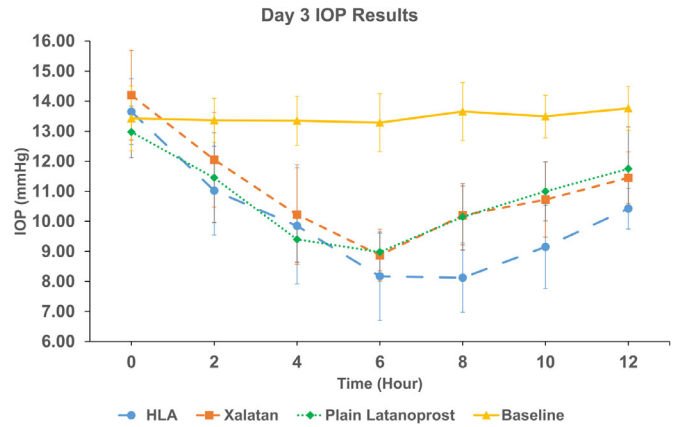
**Figure 3.** Effect of 0.005% HA-CS-latanoprost link NP eye drops on IOP at 0 to 12 hours throughout treatment days 3, 4, 5, and 6 compared w/ baseline day 2,  $**P < 0.005$ ;  $n = 8$ .

12th hour compared with those of day 2 (see Fig. 1). Whereas on treatment days 4 and 5, the IOP reduction was significantly different when compared to those of day 2 from the 2nd to 12th hours. Whereas on day 6, its IOP reduction was significantly different across all time points (0 to 12th) compared to those of day 2. See Supplementary Table S1 for the  $P$  values. On treatment days 3, 4, 5, and 6, the maximum reduction in IOP of plain latanoprost eye drops occurred at the 6th hour after each morning dose and was measured as 3.82 mm Hg (30%), 4.8 mm Hg (38%), 4.85 mm Hg (37%), and 4.25 mm Hg (33%), respectively. Whereas its mean IOP reduction on days 3, 4, 5, and 6 were 1.99 mm Hg (15%), 3.22 mm Hg (24%), 2.55 mm Hg (19%), and 2.46 mm Hg (19%), respectively. Treatment day 5 has the lowest standard deviation of 0.976 and highest on day 4 with 1.5. IOP measurements during the 9-day observation period is shown in Supplementary Table S4.

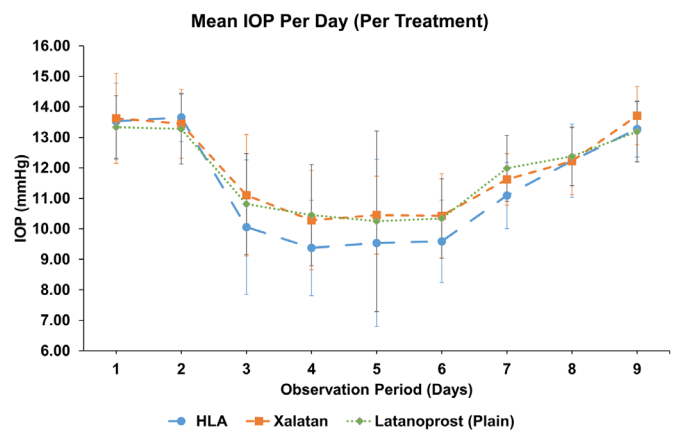
With Xalatan eye drops, reduction of IOP started also at day 3, wherein at 5% level of significance, after the first dose, IOP reduction was significantly different from the 4th to 12th hour compared to those of day 2 (see Fig. 2). On treatment days 4, 5, and 6, its IOP reduction was significantly different across all time points (0 to 12th) compared to those of day 2. See Supplementary Table S2 for the *P* values. Reduction in IOP at 0 hours before the treatment dose would indicate that the duration of IOP was at least 24 hours. On treatment days 3, 4, 5, and 6, the maximum reduction in IOP by Xalatan eye drops also occurred at the 6th hour after each morning dose and were 4.62 mm Hg (34%), 4.7 mm Hg (35%), 4.47 mm Hg (33%), and 4.8 mm Hg (36%), respectively. Whereas the mean IOP reduction on days 3, 4, 5, and 6 were 2.40 mm Hg (18%), 2.35 mm Hg (17%), 3.05 mm Hg (23%), and 3.07 mm Hg (23%), respectively. On treatment days, day 4 has the lowest standard deviation of 1.09, and the highest is at day 3 with 1.7. IOP measurements during the 9-day observation period is shown in Supplementary Table S5.

With HA-CS-latanoprost link NP eye drops, reduction of IOP started also at day 3 wherein at 5% level of significance, after the first dose, IOP reduction was significantly different from the 2nd to the 12th hour compared to those of day 2 (see Fig. 3). On treatment days 4, 5, and 6, its IOP reduction was significantly different across all time points (0 to 12th) compared to those of day 2, which would indicate that the duration of IOP was at least 24 hours. See Supplementary Table S2 for the *P* values. In days 3 and 6, HA-CS-latanoprost link NP eye drop's maximum reduction in IOP occurred at the 8th hour and was 5.22 mm Hg (39%) and 5.12 mm Hg (38%), respectively. Whereas on treatment days 4 and 5, the maximum IOP reduction was at the 6th hour, and were 5.75 mm Hg (43%) and 4.95 (37%) mm Hg, respectively. Whereas the mean IOP reduction on days 3, 4, 5, and 6 were 3.29 mm Hg (24%), 3.98 mm Hg (29%), 3.81 mm Hg (28%), and 3.76 mm Hg (28%), respectively. Day 5 has the lowest standard deviation of 0.921 and the highest is on day 3 with 1.92. IOP measurements during the 9-day observation period is shown in Supplementary Table S6.

The treatment effects of plain latanoprost, Xalatan, and HA-CS-latanoprost link NP eye drops on the IOP on the first day of treatment are shown in Figure 4. The primary focus of finding significant differences between the effect of the drugs on the IOP falls on the data gathered on the first day of administering the treatment (day 3) because the effect of the drug formulations would immediately be seen without the presence of any bias (carry-over effect, etc.). Figure 4 shows that the effect of three treatments on IOP is different



**Figure 4.** Comparison of the mean treatment effects of plain latanoprost, Xalatan, and HA-CS-latanoprost link NP eye drops on the IOP on the first day of treatment (day 3). The repeated measures were taken into account and were reflected through the error bars/standard deviation above. The three treatments are different from the baseline (control),  $**P < 0.005$ ;  $n = 8$ .



**Figure 5.** Daily mean IOP  $\pm$  SD measure of plain latanoprost, Xalatan, and HA-CS-latanoprost link NP eye drops.

from the baseline (control). Further test (the Friedman test) shows that at a 5% level of significance, there is sufficient evidence to conclude that the distributions of each treatment pairing are significantly different from each other ( $P < 0.001$ ). HA-CS-latanoprost link NP eye drops have significantly reduced the IOP compared with the other two treatments ( $P < 0.001$ ). Meanwhile, Xalatan and plain latanoprost eye drops are different from each other ( $P = 0.049$ ).

To further support the data gathered on day 3, Figure 5 shows that at a 5% level of significance, there is sufficient evidence to conclude that the average distributions of treatments are significantly different from each other across time points ( $P < 0.001$ ). The figure shows that HA-CS-latanoprost link NP eye drops have a greater IOP reducing effect compared with the other 2 treatments ( $P < 0.001$ ). The plot also shows that plain

latanoprost and Xalatan eye drops have a slight difference in distribution ( $P < 0.001$ ).

During the recovery period, where treatments were discontinued, there was an increase in the IOP measurements seen on day 7 for all of the treatment groups. At 5% level of significance, comparing recovery days 7, 8, and 9 to initial day 1, only day 9 has a distribution of IOP measurements that is significantly not different to the distribution on day 1, wherein no treatment was administered, see Supplementary Table S7. This shows that there was a gradual increase of IOP back to baseline values, which means that it would take at least 3 days of recovery for the drug effect on the IOP to diminish.

## Discussion

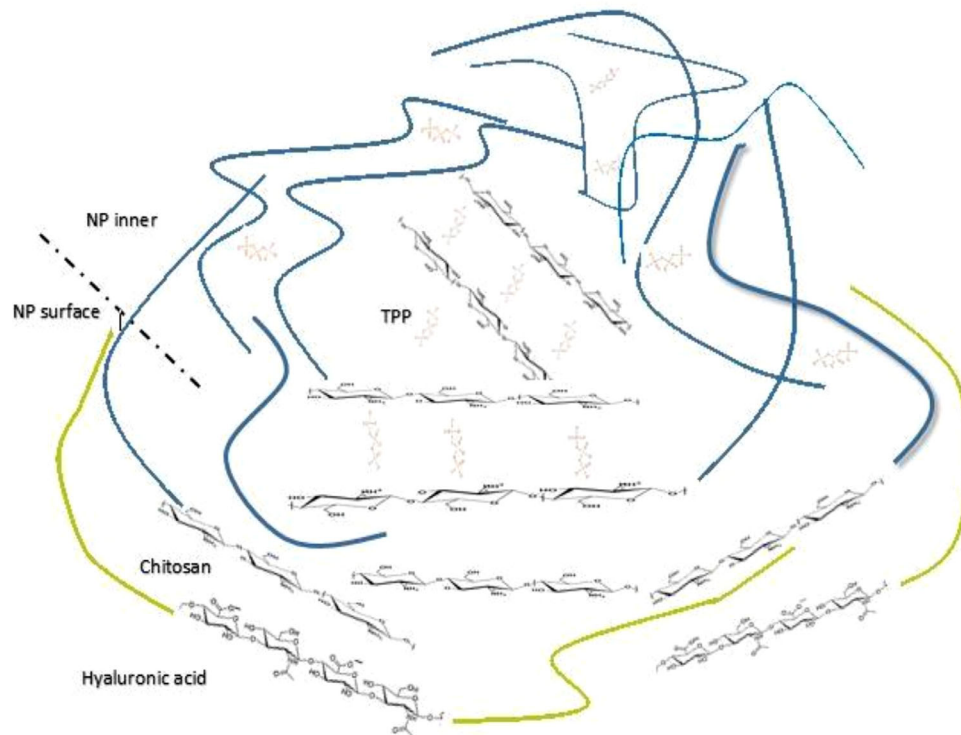
The NPs that were prepared in the study was made up of biodegradable, biocompatible, and mucoadhesive polymers, CS, and HA, and the drug, latanoprost. It is an example of a bottom-up technique where the particulate system is prepared from a state of molecular dispersion (1 nm to 100 nm), with molecular particles that spontaneously link or bind to form colloidal particles (100 nm to 500 nm). The assembly and formation of the NPs is achieved by ionic cross-linking between CS polymer chains, having partial positive charges, through anionic cross-linkers, such as TPP.<sup>10</sup>

The colloidal dispersion of CS-latanoprost link NPs was prepared by the ionic gelation method, where the negatively charged TPP serves as the cross-linker between positively charged CS molecules.<sup>34</sup> Initially, the latanoprost dispersed in water was mixed and ultrasonicated with the mildly acidic CS solution for hours. Basically, glucosamine and *N*-acetyl-glucosamine units make up the CS structure. In a solution, CS behaves as an amphiphilic polymer because of the hydrophobic nature of *N*-acetyl-glucosamine and hydrophilic nature of glucosamine.<sup>35</sup> The NPs are formed upon dropwise addition of TPP alkaline solution to the CS-latanoprost solution. TPP in alkaline solution contains hydroxyl ions and tripolyphosphate ions where these functional groups competitively bind to the amino group sites of CS. A molecule of CS has one main amino group and two free hydroxyl groups for each C<sub>6</sub> building unit. The random attachment of the hydroxyl and phosphate ions to CS result in the NP formation. Formation of NP is seen by the turning of the clear solution of latanoprost in CS solution to slightly turbid solution upon the addition of the TPP. After allowing the NPs to form, the anionic glucosaminoglycan HA was added dropwise, allowing it to attach to the surface

of the formed NPs through binding with the multi-positively charged CS.<sup>31,34,36</sup> A representation of HA-CS-latanoprost link NP is shown in Figure 6 below.

The particle size, polydispersity index (PDI) and zeta potential (ZP) of the particles in a colloidal dispersion are significant criterion for the development of ocular drug delivery systems for the prevention of eye irritation, prolonging the binding of the NPs with the corneal and conjunctival surfaces, and stability of dispersion.<sup>26</sup> In pharmaceutical liquid preparations, a colloidal dispersion represents a system having a particle size that is an intermediate between that of a true solution and a coarse dispersion, which is about 1 to 500 nm.<sup>9</sup> The NP size is significant in preventing ocular irritation, increasing ocular availability of the drug, as well as stabilizing the colloidal dispersion. Smaller particle size provides a greater surface area, which is a significant parameter for the binding of the NPs with the ocular surface, particularly the exterior of the corneal epithelial and for higher drug linking efficiency, thus it influences bioavailability.<sup>37</sup> Mucoadhesiveness is increased with high surface area due to greater interface for bonding with the mucus layer.<sup>36</sup> Suitable for ocular drug delivery is a drug loaded NPs with particle size ranging from 50 to 500 nm.<sup>31,36</sup> The Dynamic Light Scattering technique was used in determining particle size distribution. The CS-latanoprost link NP in the study had an average particle size of  $199.7 \pm 2.25$  nm, upon the addition of HA, it was increased to  $314.4 \pm 0.63$  nm. Whereas PDI measures the broadness of the particle size distribution. Uniformity of particle size or narrowness of the particle size distribution determines the stability of the dispersion of the particles in the medium.<sup>26</sup> Studies show that the PDI for CS NPs ranges between 0.17 and 1, higher than 0.7 indicates nonuniformity of particle size distribution.<sup>36</sup> The CS-latanoprost link NP in the study had a PDI value of  $0.274 \pm 0.010$ , upon the addition of HA, the PDI was increased to  $0.431 \pm 0.0121$ . Whereas the surface charge of the NPs was measured by the ZP analysis technique using Malvern Zetasizer Nano. The ZP of surface charge values that are high enough ( $\geq 30$  mV) would provide electrostatic stability and thus prevent particle aggregation that will prevent settling of particles upon storage. The repulsion of similar charges present on the NP surface prevents aggregation of particles.<sup>9</sup> The CS-latanoprost link NP in the study had ZP of  $34.97 \pm 0.757$  mV, upon the addition of HA, it was decreased to  $29.867 \pm 0.513$  mV. These surface charges are brought about by the positively charged polysaccharide CS, which binds to the negative charges found in the mucin layer of the cornea and conjunctiva along with hydrogen binding and hydrophobic interaction.<sup>8</sup> Thus, it also prolongs the corneal and

## Graphical view of the HA coated CS/TPP



**Figure 6.** The graphical representation of the HA-CS-latanoprost link NP configuration where CS amino groups are located on the ends of the TPP. The negatively charged HA molecules are bound to the surface of the positively charged CS on the surface of the NP.

conjunctival retention of NPs, that will allow the drug to have longer residence time to the anterior segment of the eye.<sup>8,36</sup>

Chitosan (CS) is a positively charged polysaccharide copolymer of 1,4-(2-amino-2-deoxy-D-glucopyranose) and 1,4-(2-acetamido-2-deoxy-D-glucopyranose), that has an amino group that binds to the negatively charged carbohydrate-bound ester sulfate ( $-\text{SO}_3\text{H}$ ) residues and carboxyl groups ( $-\text{COOH}$ ) of sialic acid in the mucin layer of the cornea and conjunctiva along with hydrogen bonding and hydrophobic interaction, making it mucoadhesive.<sup>8</sup> The mucoadhesive contact is strengthened with the interfusion across the formed interface when CS molecules interact with the mucus glycoprotein.<sup>36</sup> Aside from CS mucoadhesive properties, it can enhance the penetration of the drug it is carrying by opening tight junctions found in the epithelial cells. This binding of  $-\text{NH}_3^+$  with  $-\text{COOH}$  and  $-\text{SO}_3\text{H}$  of the mucin layer leads also to reversible structural reorganization and opening of tight junctions in the cell membrane on the mucosal surface.<sup>36</sup> The enhanced penetration of the drug leads to increased amounts of the drug to the site of action, thus providing higher accuracy of instilled drug solution.<sup>31,36</sup> The mucoadhesive property of CS and its effect to the protein-

associated tight junctions lead to the prolonged ocular residence time and increased absorption of the drug it is carrying as compared to the usual eye drops. These leads to the improvement of drug ocular delivery and availability to the site of action, as shown by the linking of latanoprost with CS NP, which leads to improved IOP lowering efficiency. Hyaluronic acid (HA) is a naturally existing anionic glycosaminoglycan with biocompatible and mucoadhesive properties that is able to interact with the ocular epithelium.<sup>38</sup> The HA also selectively binds to the CD44 and hyaluronan receptors located on the corneal and conjunctival epithelial cells, thus improving cellular targeting.<sup>26</sup> The mucoadhesiveness of CS and HA allows accumulation of the drug in the corneal epithelium, which is important for drugs like latanoprost to be significantly transported to the anterior segment of the eye.<sup>11</sup> This accumulation or deposition of latanoprost will serve as a driving force for the prolonged delivery of the drug to the site of action. The NPs without the drug may have no IOP lowering effects, thus the study came up with three treatment groups only, namely, plain latanoprost, the commercially available Xalatan, and HA-CS-latanoprost link NP formulated eye drops. The CS-drug link NPs were also used in other IOP lowering drugs for glaucoma, such as acetazolamide loaded



in CS NPs, which provide lowering of IOP in an *in vivo* study for at least 5 hours.<sup>39</sup> A study encapsulated brimonidine in ultra-small CS NPs demonstrated an *in vitro* prolonged release of the drug for 100 hours.<sup>36</sup> Another study that loaded timolol and dorzolamide into a HA-CS-latanoprost link NP showed significant IOP reduction as compared to drug treatment only.<sup>40</sup> The studies showed that the improved efficiency of IOP lowering and the sustained release were due to the mucoadhesive properties and reorganization of tight junctions in epithelial cells of CS.<sup>36</sup>

The study has compared the formulated HA-CS-latanoprost link NP eye drop with plain latanoprost and commercially available Xalatan in terms of their IOP reduction efficiency and proving the enhanced delivery of latanoprost due to linking with HA and CS as NP. Because the commercially available Xalatan is formulated with 0.02% BAC, the two other treatments have the same amount of BAC, same to that of Xalatan. The amount of the BAC in the three preparations is equal for comparability purposes.

To determine whether the IOP measurements of day 1 and day 2 could be interchanged when used as the baseline, Levene's test was used to see the equality of their variances, which resulted in having the control factor from the average measurements of the baseline day 2. At a 5% level of significance, the IOP measurements of days 1 and 2 for plain latanoprost and HA-CS-latanoprost link NP eye drops group have equal variances ( $P = 0.941$  and  $P = 0.075$ , respectively). However, the variance of day 1 is not equal to the variance of day 2 for Xalatan eye drop group ( $P < 0.001$ ). Looking at Xalatan's standard deviation of both days, day 2 has a lower standard deviation (1.21) compared to day 1 (2.34). Because the day 1 results may be considered to have unstable variances, the day 2 IOP measurements were used as the baseline for all treatments to preserve the consistency of results.

The average IOP measurements of the right eye of all test rabbits on baseline day 2 was used as control over the left eye treated with NSS, because no treatment was administered to the test animals at this period, and measurements of IOP during the treatment period came from the same right eye that was measured during the baseline period. For comparison purposes, the IOP measurements taken on day 3 from the left eye (NSS) was compared to the mean IOP measurements of day 1 and day 2 of the right eye (baseline). At a 5% level of significance, there is sufficient evidence to conclude that IOP distribution (right eye) at the baseline is significantly different from those of the NSS (left eye; Friedman test:  $P < 0.001$ ). The mean reduction in IOP measurements of NSS treated eyes (left) compared with baseline eyes (right) is 0.71 mm Hg (5%). A corresponding decrease in IOP in the left eye instilled with

NSS was possibly due to the systemic absorption of latanoprost.

The variability in IOP measurements were taken into account by having five IOP readings for each time point for both eyes of each rabbit. The standard error of the mean IOP measurement (5 repetitions) ranges around 0.5 to 1.3. The repeated measures make the standard error of the mean smaller, thus making statistical inference in the differences observed in the study. IOPs that were measured with sudden movement of the rabbits were excluded.

The rabbits have circadian rhythm of having increase IOP at night and decrease during daytime, which is opposite to those in humans.<sup>41–43</sup> The mean IOP and variances of the 24 data points per time interval (8 AM to 8 PM) during baseline day 2 are not significantly different. Thus, treatments in this study were given once daily at 8 AM to the rabbits, as opposed to the dose of latanoprost eye drops in humans that is given in the evening.

The mean values for IOP reduction during the treatment period were as follows: 27.3% ( $+/- 2.2$ ) for the latanoprost HA-CS NP (treatment group), 19.3% ( $+/- 3.7$ ) for the plain latanoprost (treatment control 1 group), and 20.3% ( $+/- 3.2$ ) for the Xalatan (treatment control 2 group). The maximum reduction of the IOP during the treatment period was: 5.75 mm Hg (43% reduction) on the 6th hour and 5.22 mm Hg (39%) on the 8th hour for the Latanoprost HA-CS NP group; 4.85 mm Hg (37% reduction) on the 6th hour for the plain latanoprost group; and 4.8 mm Hg (36% reduction) on the 6th hour for the Xalatan group during the treatment period ( $P > 0.05$ ). The daily mean IOP measurements of each treatment group showed that latanoprost HA-CS NP has greater IOP reduction effect compared with the other two treatments ( $P < 0.05$ ).

Using the Wilcoxon Signed Rank Test at a 5% level of significance, mean IOP measurements in all treatment groups are significantly different from each other on day 7 of the recovery period ( $P = 0.018$ ). The mean HA-CS-latanoprost link NP on day 7 of the recovery period was also statistically lower compared to plain latanoprost and Xalatan. Although, no more difference in IOP measurements between treatment groups was observed on day 8 of the recovery period.

In a journal published in 2013, bimatoprost, another PGF<sub>2α</sub> analog, when formulated in Durasite (polycarophil based polymer) provided an improved pharmacokinetic parameters compared to conventional eye drops; such as superior ocular distribution and bioavailability, as well as less eye side effects due to long-term use of prostaglandin analogues.<sup>44</sup> Bimatoprost was also formulated into solid lipid nanoparticles, its *in vitro* and *ex vivo* studies demonstrated prolonged

drug release.<sup>45</sup> Another PGF<sub>2α</sub> analog, travoprost was loaded to lipid DNA NPs where its ex vivo and in vivo studies showed that the quantity of travoprost delivered doubled the amount of the drug as compared to the pristine drug.<sup>46</sup> Latanoprost was developed into liposomal Pluronic gels for sustained ocular delivery, achieving a prolonged and superior reduction in the IOP for up to 3 days, which was significantly longer than the conventional eye drops, thus reducing frequency of instillation and improving patient compliance.<sup>47</sup> In another study, latanoprost was prepared into liposomal formulation administered by subconjunctival injection providing sustained release of the drug for 50 days and was comparable to the efficiency of daily instillation, thus significantly beneficial over the eye drop formulation.<sup>48</sup> It was also formulated into multi-layered nanosheets in another study that is made of CS and sodium alginate, providing a sustained release over a week with reduced IOP, after a single placement of the nanosheet on the corneal surface.<sup>13</sup> The researcher's study together with the studies mentioned above showed that PGF<sub>2α</sub> analogs, when formulated with mucoadhesive NPs, their quantity and delivery to the site of action may increase and be prolonged.

In this study, the significant reduction in IOP seen in the formulated HA-CS-latanoprost link NP eye drops could be attributed to the CS association with the latanoprost, which extends the drug's contact time to the ocular site. Furthermore, the adhesion of CS to the mucus layer will allow the drug's drainage to be controlled by the mucus turnover time, which is approximately 15 to 20 hours rather than the tears, which is approximately only 15 to 30 seconds, which is longer, therefore, providing prolonged corneal retention.<sup>11,26</sup> CS offers an attractive benefit for the treatment of chronic ocular diseases, like glaucoma, because of its property of intimately adhering to the corneal and conjunctival surfaces that can form a precorneal depot resulting in a prolonged release of the bound drug. Although HA also improves the effect for mucoadhesion and selectivity, in association with CS.<sup>25,27,49</sup>

According to epidemiological studies, NTG represents 50% or more of all open-angle glaucoma all over the world.<sup>50</sup> Even though high IOPs are absent in NTG, the normal levels of IOP still leads to optic nerve damage, thus, PGF<sub>2α</sub> are used in patients with NTG.<sup>50–52</sup> IOP is involved in NGT, which confirms that it is involved in the pathogenesis of NGT.<sup>7,51</sup> This is one of the reasons why the study was performed in normotensive rabbits and the IOP reduction activity was measured. Where the positive results of the study may serve as a basis that the IOP reduction activity may further be tested to glaucoma induced rabbit

models, thus the study recommends to perform the test on intense IOP elevation ocular hypertensive rabbit models.

The IOP reducing test shows that the formulation of latanoprost with CS and HA is more effective in reducing the IOP than by drug alone in normal rabbits. These results could serve as a basis that HA-CS-latanoprost link NP formulation could improve and sustain drug concentration in the active site, thus could aid in the formulation of latanoprost eye drops with decreased or no benzalkonium HCl as an irritant preservative and penetration enhancer. These characteristics of drug delivery are an advantage for eye drops especially those for long term use, such as glaucoma medications, because it could improve patient compliance as well.

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