Original Article

Evaluation of Chitosan; a Thermosensitive Hydrogel Drug Delivery Agent for Loading Dexamethosone

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Purpose: To evaluate the characteristics of thermosensitive hydrogel (Chitosan) drug delivery agent loaded with dexamethasone/chitosan which can be used in the treatment of macular edema and non infectious uveitis applied in sub tenon space.

Study Design: Quasi experimental study.

Place and Duration of Study: Lahore General hospital, IRCBM COMSATS Institute of Information Technology, Lahore, Pakistan from July 2017 to July 2018.

Material and Methods: Acetic acid (0.5 M, 2.5 ml) and Chitosan (0.2g) were dissolved and stirred for 1 hour and 30 minutes at room temperature. Powdered dexamethasone (3.5 mg) was added and stirred for further 30 min at room temperature. This solution was placed at 4°C for 30 min to cool it down. After this, $NaHCO_3$ solution (0.48 M, 2 ml) was added drop wise. Once required pH was achieved, solution was placed inside oven at 37°C. Formation of gel started after 3-5 minutes and it took 2 hours for complete conversion of liquid into hydrogel.

Results: The gelling time of the synthesized gel was 2 hours and was tested by test tube invert method. To prove the non-irritancy of thermosensitive hydrogel, Hens Egg Test on Chorioallantoic membrane assay (HET-CAM) was performed. Results showed that synthesized hydrogel was non-irritant. Outcomes

of *in vitro* degradation tests displayed that synthesized hydrogels were biodegradable. The drug release tests revealed that synthesized hydrogels displayed sustained release of drug.

Conclusion: The analysis showed that physical changes and drug loading did not alter the chemical structure of chitosan therefore it is an effective potential vehicle for slow release of Dexamethasone if placed in the sub tenon space.

Keywords: Chitosan, dexamethasone, macular edema, non-infectious uveitis.

To treat posterior segment eye diseases, four routes are available i.e., topical, periocular, intraocular and systemic. Topical and systemic routes are not preferred route of drug administration because of having some significant disadvantages i.e., low ocular bioavailability of drug, frequent administration of high amount of drugs[1].

Periocular route is the most preferable route for instillation of drug to the posterior segment of eye[2] ensuring higher retinal and vitreal drug bioavailability (0.01-0.1%) which is higher than topical medication (≤ 0.001%)[3]. Sub-tenon route is most preferable periocular route. The disadvantages of this route include cataract, hyphema, corneal decompensation and rise in intra ocular pressure. Disadvantages of intravitreal steroid injections are worse than periocular sub-tenon pathway. Therefore, sub-tenon route is preferred pathway for administration of steroid[4].

Intravitreal injections have earned fame among researchers and clinicians. Unlike topical and systemic routes, it offers high concentration of drug to vitreous, retina and choroid[5]. Though it ensures bioavailability of drug, instillation of drug through this pathway is invasive and potentially risky which causes endophthalmitis, retinal detachment and vitreous hemorrhages[6].

Scientists have made many efforts to enhance bioavailability of drug by designing different drug delivery systems i.e. ointments, suspensions, gels, collagen shields, implants and hydrogels[7]. In general there are three types of implants available in market for treatment of posterior segment eye diseases: non-biodegradable, biodegradable and stimuli responsive implants. In non-biodegradable implants, FDA has approved Vitrasert® and Retisert®. Former implant carries ganciclovir drug for treatment of Cytomegalo virus retinitis. It releases drug for 8 months. While the later implant carries fluocinolone acetonide to treat chronic non-infectious posterior uveitis. Iluvien® implant is waiting for FDA approval but accepted in some EU countries. For degradable implants, FDA has approved only Ozurdex®. None of these implants are available in Pakistan for treatment of patients suffering from posterior segment eye diseases. However, each system has its own advantages and disadvantages.[8]

Among all such devices, in-situ forming hydrogel has gained enormous attention by scientists. These hydrogels are liquid at room temperature and solid under physiological conditions[9]. These in-situ hydrogels can be achieved by several ways such as pH change, ionic cross linkage and temperature modulation. Among all these, thermosensitive hydrogels got immense attention for ocular treatments because of its easy handling and low viscosity at room temperature[10,11].

Chitosan, (poly-β(1,4)-D-glucosamine), has been extensively used as implant in the form of gels, fibers and membranes in the field of tissue engineering and biomedical sciences and drug controlled release systems. Since chitosan is highly biocompatible, therefore, it has been extensively used for the synthesis of thermosensitive hydrogels which help to treat ocular diseases. Various drugs have been loaded on to chitosan based thermosensitive hydrogels for treatment of ocular diseases for example, latanoprost was loaded on chitosan and gelatin based thermosensitive hydrogel for controlling ocular hypertension[12]. Chitosan in combination with disodium α-d-glucose 1-phosphate (DGP) has been used for ocular drug delivery system[13]. A novel copolymer, poly (*N*-isopropylacrylamide)–chitosan (PNIPAAm–CS), was investigated for its thermosensitive in situ gel-forming properties and

potential utilization for ocular drug delivery[14]. Another novel thermosensitive hydrogel was made by using chitosan and glycidyltrimethylammonium chloride (GTMAC) and named as *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC)[15].

Chitosan has been used as a carrier of dexamethasone drug to treat ocular diseases i.e., Mucoadhesive chitosan-coated cationic microemulsion of dexamethasone for ocular delivery[16,18]. Keeping in mind all such information, in present work, we are for the first time, aiming to use biodegradable chitosan thermosensitive gel loaded with dexamethasone and finding the potential use of sub-tenon space for insertion of these synthesized gels. This will provide sustained release of drug and will overcome the side effects of previous treatments to treat posterior segment eye diseases especially macular edema and uveitis. The cost of pre-existing treatments of these diseases are expensive and sometimes unaffordable when considering needs of individual patient.

MATERIAL AND METHODS

Chitosan (DD = 80.91% and Mol. Wt. = 25992.88) was synthesized in our laboratories. Acetic acid (CH_3COOH) was purchased from Riedel-deHaen (origin). From Bio world (origin) PBS (Phosphate Buffer Saline) was bought. $NaHCO_3$ was bought from Daejung chemicals and metals CO., LTD (Korea). Dexamethasone was bought from Zhejiang Xianju Junye pharmaceutical Co., Ltd (China). NaCl was obtained from Omicron sciences LTD (UK). From Sigma-Aldrich (Germany) sodium hydroxide (NaOH) was bought.

In acetic acid (0.5 M, 2.5 ml) Chitosan (0.2g) was dissolved and stirred for 1 hour and 30 min at room temperature. Powdered dexamethasone (3.5 mg) was added and stirred for further 30 min at room temperature. This solution was placed at 4°C for 30 minutes to cool it down. After this, $NaHCO_3$ solution (0.48 M, 2 ml) was added drop wise. In the meanwhile, pH change was monitored and finally maintained at 7. Constant stirring was done to remove effervescence. Once required pH was achieved, solution was placed inside oven at 37°C. Formation of gel started after 3-5 minutes and it happened from the surface first. It took 2 hour for complete conversion of liquid into hydrogel.

Test tube invert method was used to analyze sol-to-gel transition. In this method, 0.5 ml polymer solution of given concentration was taken in 3 different vials. The vials containing polymer solution were placed at 4°C for 30 min – 1 hour. After this, each vial was immersed in separate water bath having different temperatures i.e. 10°, 25° and 37°, for 10 min. After 10 mins, each vial was taken out and inverted to 180°. If no visible flow was observed within 30s of inversion, sample was considered as "gel".

The pH of sample before and after gelation was calculated by calibrated pH meter (Eutech instrument pc 150). Neutral pH is the indication of completion of reaction between acid and base which is required for conversion of sol-to-gel. Also, acidic or basic gel implant can cause irritation to the eye and can permanently damage the tissue. Therefore, it is important to measure the pH of the gel.

Structural characterization of prepared thermosensitive hydrogels was carried out by Fourier transfer infrared (FTIR) spectroscopy, coupled with smart ATR accessory. Thermo-Nicolet 6700P FTIR Spectrometer (USA) was used and the average number of scans were 256 at the resolution of 8 cm^{-1}. Spectra that were recorded ranged in wavelength of 4000-650cm^1.

Scanning electron microscope (Tescan, Vega LMU) at 10 kV under low vacuum mode at 10 Pa was employed for the assessment of pore size and compact structure of synthesized hydrogel. At different magnifications images were obtained. Image processing software (Image J) was used to calculate the diameter of pore by selecting 30 pores randomly.

For every sample composition (n=3) degradation tests were performed gravimetrically. Two weights were taken to achieve this purpose. Before immersing them into solutions, initial dry weight (W_1) of hydrogels was taken. Then the samples were kept in phosphate buffered saline (PBS), lysozyme solution (1mg/ml) in PBS at 37°C for different time points (day 1-day 28). The samples were taken out at each time point, dried at 37°C for 24 H and subsequently weighed (W_2). The dried weight (without water content) remaining ratios were determined as following:

$$Dry\ weight\ remaining\ ratio\ (\%) = \frac{W_2}{W_1} \times 100$$

Drug release test was carried out in PBS. For this purpose, powdered dexamethasone was added in

stirring solution of chitosan and sodium bicarbonate wherein drug content was 0.7 mg/ml. After adding drug, resultant mixture was placed in oven at 37°C to form gel. Hydrogel was cut into triplicates of equal weight (10 mg) and dipped into 5 ml PBS solution. At different time intervals (after 3 h, after 16 h, after 24 h and after 48 h) PBS solution in vials was replaced with the fresh one. Collected PBS solutions were analyzed under UV/Visible spectrophotometer (Perkin Elmer). Amount of drug release was determined by following straight line equation:

$$y = mx + c$$

HET-CAM (Hen's egg test – chorioallantoic membrane) assay, most robust and successful assay, was used to evaluate irritation properties of chemicals and consumer products that might come in contact with human eyes. The assay covers a broad spectrum of chemicals with whole range of degrees of irritation and physical appearances of different substances. To evaluate the ocular tolerance of the developed thermosensitive hydrogel, HET-CAM test was performed with small modifications.

Briefly, freshly fertilized hen's eggs were bought from Big Bird Group (Lahore, Pakistan). They were put in an incubator at 37.8 ± 0.5°C and 55% humidity for nine days. At day 10, the egg shell was opened, and white egg membrane was removed carefully without injuring any underlying blood vessels. Subsequently, the surface of the CAM was exposed to 0.1g of the test substance, 0.1 M sodium hydroxide (NaOH) solution (positive control), and a 0.9% NaCl w/v saline solution (negative control). The chorioallantoic membrane and its clearly delineated vascular system was further assessed subjectively in terms of hyperemia, hemorrhage or coagulation. Changes were examined using a light microscope (Mitotic, China) before exposure and at different time points post-application for 5 min. Scoring of each test substance was designated by using a classification system previously described by Luepke and Kemper (1986): Non irritation: up to 0.9; slight irritant: 1-4.9; moderate irritant: 5-8.9; severe irritant: 9 and above. Moreover, images were obtained before application and for 30 s, 2 min, and 5 min after exposure.

RESULTS

In the preparation of Thermosensitive Hydrogel of Chitosan and loading with Dexamethasone, it was shown that neutralization occurred which resulted in the formation of physical junctions (hydrogen bonding) between polymeric chains of chitosan.

In chemical structure analysis by Fourier Transform Infrared Spectroscopy it was shown that only temperature changed the physical appearance of polymer which was not significant.

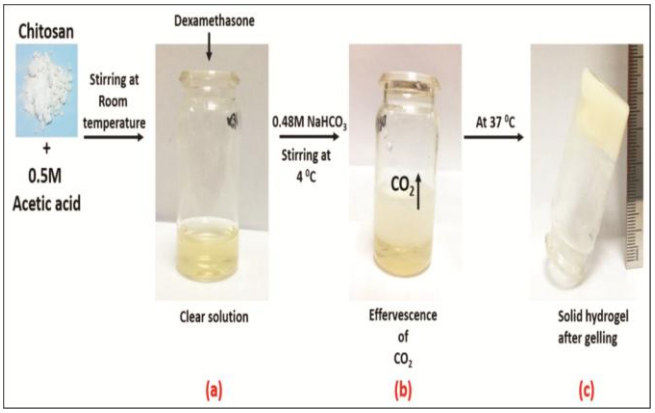


Fig. 1: *Step by step illustration of synthesis of thermosensitive hydrogel.*

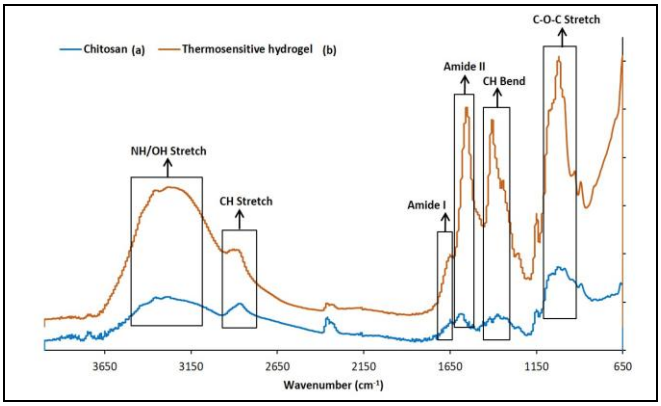


Fig. 2: *FTIR results of pure chitosan (a) and thermosensitive hydrogel (b).*

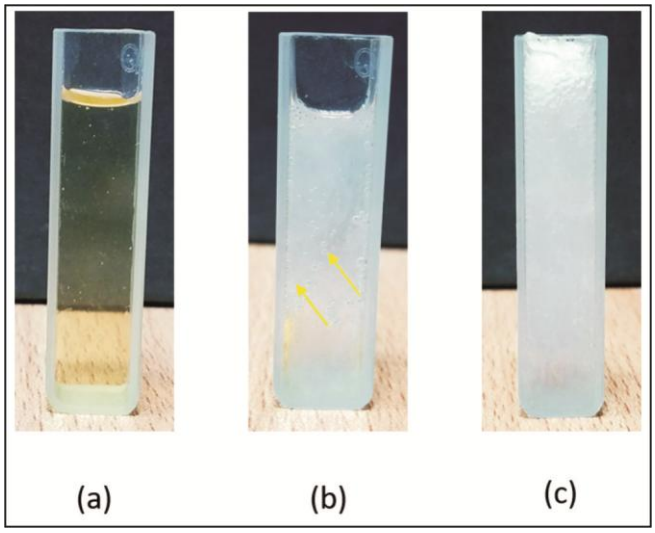


Fig. 3: *Sequence of pictures illustrating physical changes occurred at 37°C. (a) Clear solution mixture before gelation. (b) Gelation started at 37°C. (c) Gelation completed at 37°C. Yellow arrows are indicating effervescence of CO_2.*

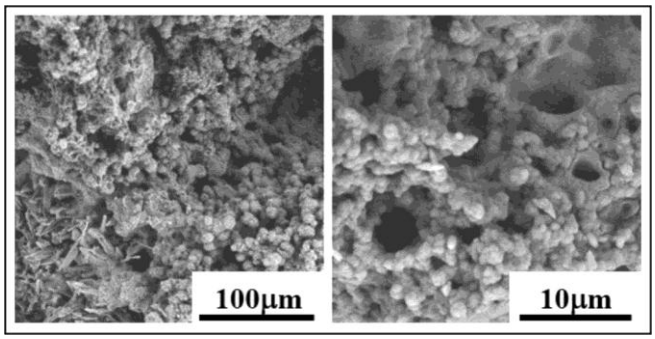


Fig. 4: *Scanning electron micrographs of synthesized hydrogel (magnification bars are given with each image).*

Characterization of Sol-to-Gel transition temperature by Test-Tube invert method showed that no CO_2 was released at 4°C hence no gelation occurred. At 25°C, no gelation was observed within 10 minutes. But after immersing vial for 2 h, gelation started at very slow rate. Best results were obtained at 37°C. Before gelation the pH was 4.9 and after gelation it was 7.14.

In-vitro drug release results showed that hydrogel can stay in sub-tenon region of eye over a month and release drug. Based on these results, we are proposing that our synthesized biomaterial will be the first thermosensitive chitosan based hydrogel which will support sustained release of dexamethasone in sub-tenon region of the eye.

To assess degradation potential of synthesized biomaterial, in vitro degradation test were performed.

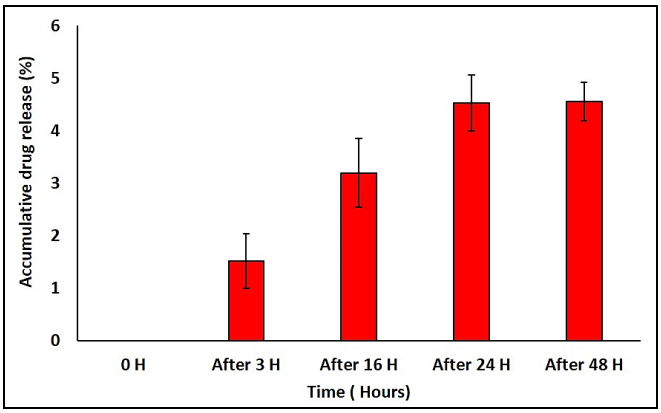
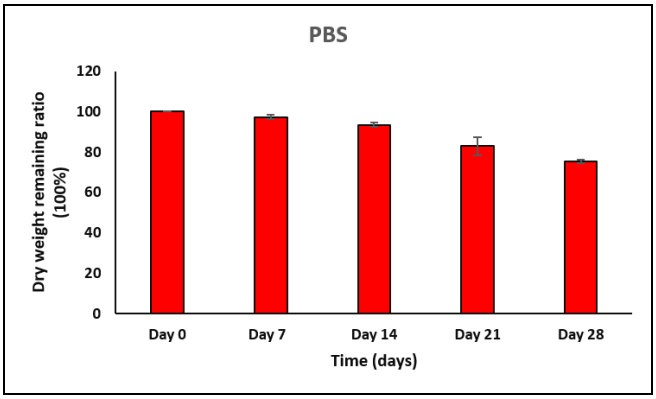


Fig. 5: *In vitro accumulative release of dexamethasone from chitosan hydrogel.*



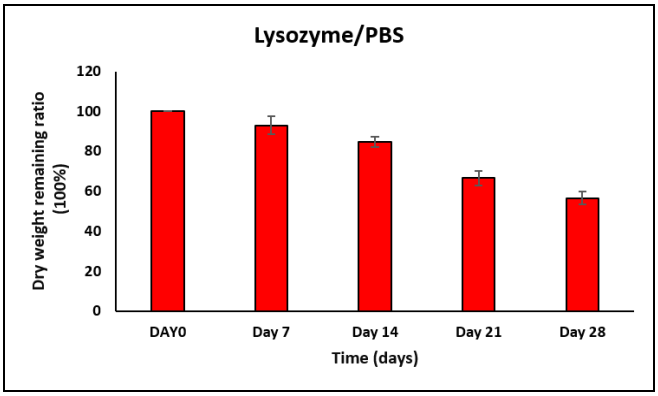


Fig. 6: *In vitro degradation in PBS and Lysozyme.*

We managed to mimic the physiological environment by selecting two media for degradation; PBS and lysozyme. From the results, it was concluded that

synthesized materials were degradable. PBS and lysozyme, both caused degradation to synthesized hydrogels. Statistical analysis revealed significant difference ($p = 0.0005$) between the degradation values of day 7 and 28.

In ocular irritancy test by HET-CAM which is a semi qualitative test to asses the irritation potential of a testing material. From figure 8, it was concluded that synthesized thermosensitive hydrogel was not irritant.

DISCUSSION

In the preparation of Thermosensitive Hydrogel of Chitosan and loading with Dexamethasone, the gelling mechanism of solution mixture of chitosan at 37°C involves neutralization of chitosan solution in the presence of $NaHCO_3$ (Figure 1). When chitosan is dissolved in 0.5M acetic acid solution, protonation of amino groups of chitosan takes place. At this point the pH of solution is 4.9. As 0.48M of $NaHCO_3$ solution is added into the 0.5M of chitosan solution, CO_2 evolves. By experimentation, it is concluded that this neutralization reaction occurs only at or above 37°C[19].

In chemical structure analysis by Fourier Transform Infrared Spectroscopy, FTIR spectra are obtained for pure powdered chitosan and synthesized thermosensitive hydrogel. Broad peak between 3200-3500 cm^{-1} appears due to NH/OH stretching vibrations. The absorptions present in the range of 2919-2910 cm^{-1} are assigned to CH stretching vibrations and peaks for CH bending vibration were present around 1400 cm^{-1},. The absorptions around 1650 and 1585 cm^{-1} are attributed to amide I (-C = O stretch) and amide II (-C-N stretch and -C-N-H bending vibrations), respectively[20]. It is found that C-O-C deformation band appears around 1097cm^{1} Characterization of Sol-to-Gel transition temperature by Test tube invert method is used to analyze the temperature required for sol-to-gel transition. For this purpose, 5 mL of fresh chitosan/$NaHCO_3$ mixture having dispersed dexamethasone is added into vial. This vial is immersed in water bath for 10 minutes at three different temperatures: 4°C, 25°C and 37°C. Gelation time is observed by tilting vial at an angle of 90° for 1 min till no flow.

From results it is observed that no CO_2 is released at 4°C hence no gelation occurs. At 25°C, no gelation is observed within 10 minutes. But after immersing

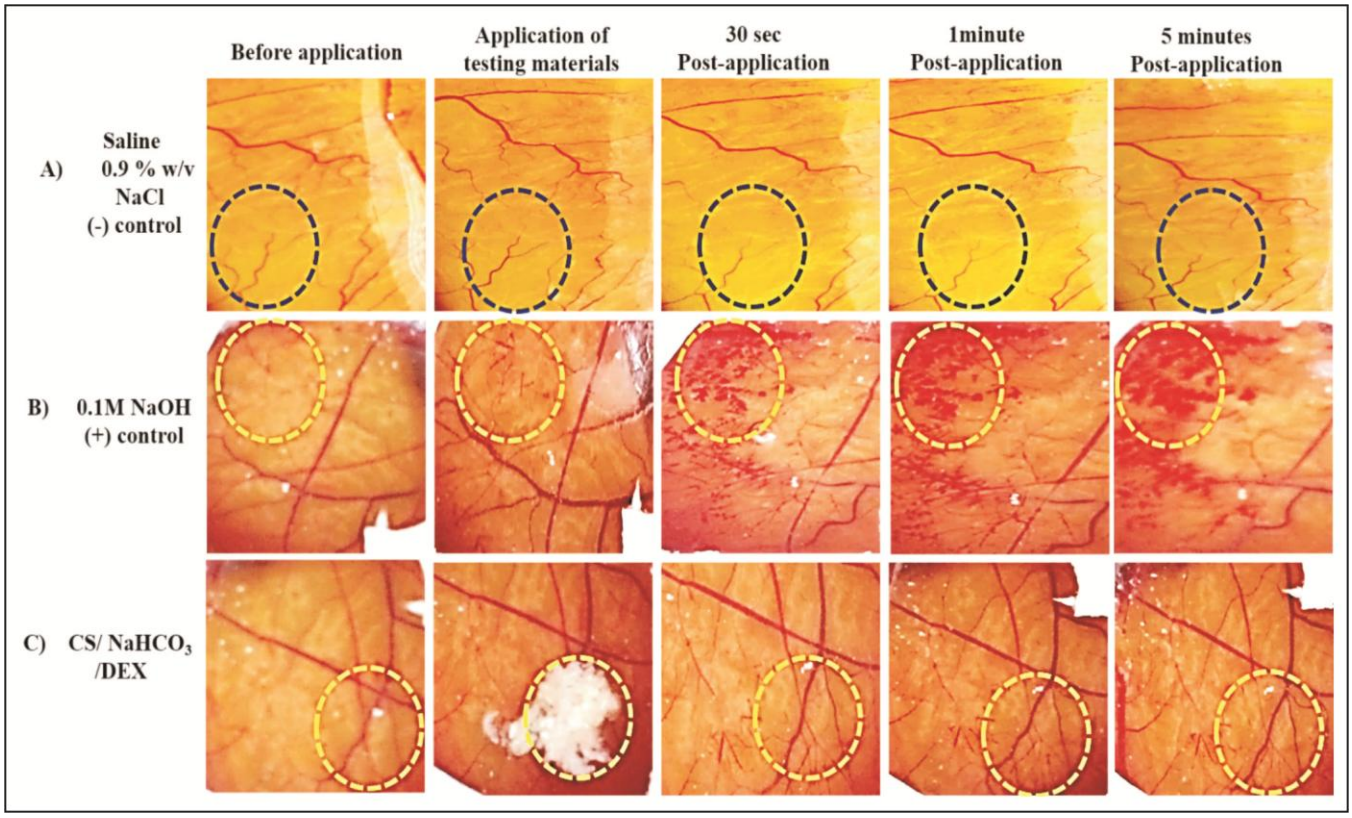


Fig. 7: *Sequence of photographs of HET/CAM test illustrating the effect of A) Saline 0.9% w/v, B) 0.1M NaOH, C) Chitosan/$NaCO_3$/DEX.*

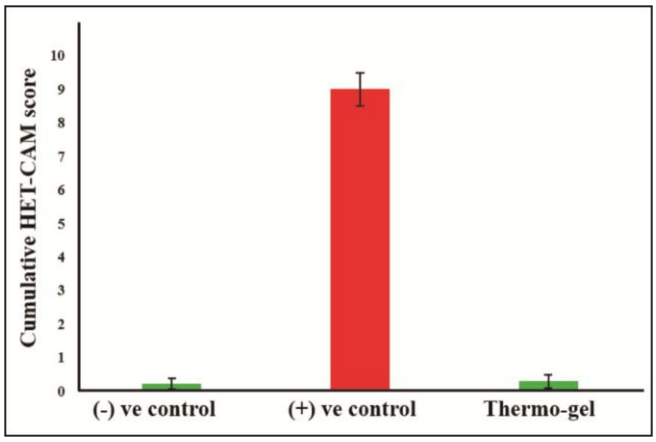


Fig. 8: *Cumulative HET-CAM score: 0.9% saline solution (NaCl 0.9% w/v) (-ve control); O.1M NaOH (+ve control); Synthesized thermo-gel.*

vial for 2 h, gelation starts at very slow rate. Best results are obtained at 37°C. As solution mixture in vial gains 37°C temperature, gelation starts instantly but completes after 2 h. The reason is quick liberation of CO_2 which results into neutralization of solution mixture and conversion of liquid solution into solid hydrogel[21].

In addition to other factors, drug release from hydrogels depends upon pore structure and pore size of hydrogel. The porous nature of biomaterial assists in high loading capacity and controllable release of drug.

To analyze porous nature of synthesized dried hydrogels, Scanning Electron Microscopic (SEM) technique is employed. SEM images show that pores and void spaces are present and are very well connected with each other. The mean pore size of hydrogels is: 31.7913 µm ± 2.855µm.

From literature, it is confirmed that 30 µm pore size in chitosan based thermosensitive hydrogel provides sustained release of drug for ocular diseases treatment.

Half-life of dexamethasone is shorter than other corticosteroids[22]. Therefore, sustained and continuous release of dexamethasone is important. From this

current research the cumulative release of dexamethasone is shown in figure 6. It is concluded that hydrogel exhibits sustained release of drug. It provided only 5% release of dexamethasone over time span of two days. This sustained release of drug may be attributed to the inner dense network of hydrogel which traps the drug through hydrogen bonding causing slow release of drug[23]. Results show that hydrogel can stay in sub-tenon region of eye over a month and release drug.

Degradation studies are performed for 28 days. Lysozyme is taken because it is confirmed from literature that it is present in specific quantity in various ocular inflammations. To investigate the biodegradation of polymeric scaffolds, Lysozyme, a renowned enzyme is used for cleavage of carbohydrates. *In vitro* studies of chitosan, Lysozyme have been used extensively as it breaks 1, 4-β linkage of carbohydrates, disassociate them[24]. Lysozyme is taken as 0.0068 mg/ml that corresponds to the concentration of lysozyme in human eye.

Potential of irritation can be detected by observing changes to the delicate vasculature of chorio allantoic membrane which is similar to the vascularized mucosal tissue of human eye.

Scoring of irritancy potential is classified according to Luepke and Kemper (1986)[25]. To compare the results, 0.1M NaOH is used as positive control and 0.9% (w/v) NaCl solution is used as negative control. NaOH caused flower like bursting, hemorrhaging and swelling on CAM. The score of irritancy potential is recorded as 9.

Saline solution and thermosensitive hydrogel does not cause any bleeding, swelling or hemorraging. Scoring of irritancy potential is recorded as 0.23 for negative control (saline solution) and 0.3 for tested thermogel. Hence, we conclude that synthesized hydrogel will not cause any harm to eyes.

CONCLUSION

In current study, a biodegradable, non-irritant and an inexpensive injectable thermosensitive gel was prepared to use in the sub-tenon's space for sustained release of dexamethasone. This study was supported by performing various tests: FT-IR confirmed that chemical structure of thermogel was not altered by dexamethasone, in vitro degradation studies exhibited 24.52% degradation in PBS solution and 43.45% in lysozyme solution, in vitro drug release studies confirmed sustained release of dexamethasone from thermogel and HET-CAM assay helped in assessing irritancy potential of prepared hydrogel confirming their non-irritant behaviour.. The synthesized hydrogel is a promising economical vehicle for sustained release of dexamethasone to the posterior segment of eye and efficient alternative of existent costly procedures.

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