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Original Article

A pH-Responsive Psyllium-Hyaluronic acid and Collagen based Hydrogel for Oral Insulin Delivery

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ABSTRACT

Diabetes mellitus is a chronic and heritable condition which grows adverse with date and eventually accelerates numerous difficulties such as end-stage renal disease, cardiac infections and vision problems. **Objective:** To highlight protein-based hydrogels as a contemporary focus in insulin delivery through the oral cavity. **Methods:** Novel hydrogels were formed in this study by using biomaterials (Psyllium, Hyaluronic acid and Collagen). The hydrogels were synthesized through a methodical process involving the combination of psyllium ispaghol husk, hyaluronic acid, and collagen for targeted insulin delivery. Initially, 1g of psyllium ispaghol husk was uniformly mixed with 200ml of distilled water. After achieving a homogeneous swelling, hyaluronic acid and collagen were added to the mixture. The synthesis of hydrogels was achieved by allowing the mixture to incubate at 38°C O/N. Physical characterization was done using FTIR analysis which indicates different bonding patterns. **Results:** Swelling ratio and drugs kinetics of hydrogels reveal maximum swelling and drug release at alkaline pH while minimum at acidic pH. Swelling kinetics shows that hydrogels followed less Fickian diffusion. These points favour the delivery of insulin in the intestine while escaping the acidic medium of stomach. Zone of inhibition around the hydrogels illustrated its antimicrobial activity. Finally, its administration to mice indicates the delivery of insulin by the decrease in glucose level measured by glucometer. **Conclusions:** Based on the diverse analyses conducted, it can be inferred that utilizing biomaterial-based hydrogels holds significant promise for effective insulin delivery through the oral route, especially beneficial for diabetic patients.

INTRODUCTION

Diabetes mellitus, characterized by irregularities in insulin production and action, represents a spectrum of metabolic disorders. The inability to produce or utilize insulin results in metabolic abnormalities of carbohydrates, lipids and proteins. It increases the rate of glucose transport across the cell membrane, and inhibits lipolysis and protein degradation. Based on factors like insulin production, utility and glucose intolerance, Diabetes manifests in three major types: type 1 and 2, third is gestational diabetes . The duration and type of diabetes determine the severity of symptoms and if not treated may lead to stupor, comma or even death. Regular exogenous administration of insulin is required for type 1 diabetes and in the advanced phase of type 2 diabetes. For effective diabetes control and healthy glycemic level, patients may require to administer two to four daily insulin injections, adding up to several thousand injections throughout their lifespan . The conventional approach to administering insulin is by subcutaneous

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injection that is painful for patients receiving multiple doses daily. Although the ultimate goal is to completely abolish the use of exogenous insulin and make the patient regain the ability to produce and use insulin, several alternatives have been tested to ease the patient's life. Recently the use of hydrogels has been proposed to be an effective alternative strategy for administering insulin orally . Stimulus-responsive hydrogels are of paramount significance due to their ability to undergo reversible changes triggered by environmental cues, including shifts in pH, temperature, and ionic strength. The pH-responsive hydrogels can be utilized for the delivery of insulin at specific targeted sites in the small intestine while being protected from the acidic environment of the stomach [4]. Limitations associated with the hydrogels include low tensile strength which might result in premature dissolution, limited capacity and homogeneity of drug loading. Furthermore, high water content and large pore size might leads to rapid drug release. With the advancement in polymer engineering and organic chemistry, hydrogels can be tailored for the targeted drug delivery, specifically to protect insulin from enzymatic degradation, and for the sustained release to maintain prolonged drug concentrations [5]. Hydrogels are 3-D cross-linked polymeric networks that are composed of hydrophilic monomers and have the capacity to imbibe significant water content. A broad spectrum of chemical compositions and physical properties can be achieved by utilizing an array of water-soluble polymers [6]. Polymeric networks in hydrogels require specific crosslinking (Physical or chemical) to prevent premature dissolution. Also, the chosen crosslinking impacts the hydrogel properties based on the intended application. In physical linkage, polymeric chains are cross-linked by hydrogen bonding, hydrophobic interaction or chains entanglement while in the case of chemical linkage, the bonding can be covalent [7]. The potential toxicity concerns related to chemical crosslinking can be addressed by removing cross-linkers before usage. Dual-network (DN) hydrogels (a mixture of chemical and physical linkage) possess better swelling capacity and are more responsive to pH variations. The high porosity of hydrogels which can be altered by varying cross-links density allows their use as pharmaceutical excipient [4]. Their performance can be optimized by making them biocompatible, self-degradable, and stimulus-responsive. Owing to these unique properties hydrogels finds various applications in cell culture and targeted drug delivery [8]. Psyllium-based hydrogels provide the double potential for oral insulin delivery because psyllium interferes with the absorption of glucose in the intestine. The husk of psyllium seeds provides mucilaginous polysaccharides which help in hydrogel formation. The mucilaginous features of psyllium husk give therapeutic applications for psyllium-based hydrogels because it can absorb water giving lubrication [9]. The amount of water penetrating in a psyllium-based hydrogel is the crucial aspect in defining the diffusion and absorption of different solutes/drugs from the hydrogel. In addition to psyllium, collagen and hyaluronic acid (HA) hold significant promise as constituents for hydrogels [10]. These components represent vital constituents of the extracellular matrix (ECM), making their integration into hydrogels advantageous as it mirrors the natural ECM in both structure and function. Collagen, a primary protein in the ECM, serves as a crucial point of interaction for cells through various cell surface receptors like integrin [8]. It is easily obtainable from a variety of tissues and animals, making it readily accessible for both investigation and medical uses. HA, on the other hand, is greatly hydrated glycosaminoglycan extensively present in the ECM of various tissue types. It plays an essential part in several biological procedures and tissue functions. The acetyl, hydroxyl and carboxyl are diverse functional groups of HA, facilitate chemical modifications, resulting in HA-based hydrogels with versatile properties [11]. In the current study, we have synthesized the hydrogels by combining the psyllium, hyaluronic acid, and collagen, and evaluated its potential for the targeted delivery of insulin. In order to achieve cross-linking, glutaraldehyde (cross-linker) and initiator (ammonium persulfate, APS) were used. The engineered hydrogel offers protection against physiological destabilization and degradation within the low-pH environment of the gastrointestinal tract.

METHODS

Husk of Psyllium plant was procured from Qarshi Industries (Lahore, Pakistan). Ammonium persulfate (APS) was obtained from Sigma Aldrich Pakistan; Cross-linker was procured from Bio Basic Inc. Hyaluronic acid (HA) was isolated from the E coli strain JM 109 strain. Swim bladders were purchased from the local market, and Humulin 70-30 (insulin) was purchased from Lilly. Collagen was isolated from chicken heart purchased from local market of Lahore. For the isolation of HA, 200µL of E. coli strain JM 109 strain was used to inoculate 100ml of Luria-Bertani (LB) media. It was incubated at 37°C for 48 hours. After that, the broth was diluted with 0.1% SDS equal to the volume of broth. It was incubated at room temperature for 10 minutes. Next double volume of chilled ethanol was added to precipitate HA. It was then incubated at 4°C for 1 hour. HA precipitate was collected by centrifuging the solution at 300 RPM for 20 minutes at room temperature. The precipitates were resuspended in 0.1M NaCl and stored at 4°C for next time use [12]. For the isolation of collagen, Non-collagenous

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proteins and fats were removed from the chicken heart by soaking them in 0.2M NaHCO3 for 3 hours at 4°C. After 4 hours they were neutralized by washing with water and checking the pH after regular intervals. When they attained pH 7 they were treated with 0.5M lactic acid (1:10 w/v) for 24 hours at 4°C. After 24 hours supernatant was collected by passing the sample through muslin cloth and collagen in the supernatant was precipitated by adding 2.5M NaCl (1:1 v/v). The precipitate was collected by centrifuging the mixture at 10,000 rpm for 20 minutes at 4°C. Isolated collagen was resuspended in 0.1M acetic acid (1:3 w/v). Uronic acid carbazole test and FTIR analysis were performed for the confirmation of HA while SDS PAGE analysis was performed for collagen. Uronic acid carbazole test was performed in which 0.25ml of the sample isolated was mixed with 1.5ml chilled sulphuric acid reagent (SAR). It was incubated in a boiling water bath for 20 minutes and then instantly cooled on ice. 50µl of Carbazole reagent was added and the mixture was incubated again in a boiling water bath for 15 minutes. In this experiment distill water was used as the negative control while D-Glucuronic acid was used as a positive control [13]. Fourier Transform Infrared (FTIR) spectroscopy was conducted on both the original molecule and the framed hydrogel using Shimadzu IR Prestige-21 in Kyoto Prefecture, Japan. The resolution power was set at 4 cm-1 within the wavenumber range of 500-4000 cm-1. 8% SDS PAGE was performed for the analysis of collagen as described in the Sambrook and Russel manual. Hyaluronic acid (HA) was quantified by using near-infrared spectroscopy in transmission mode according to the protocol described elsewhere [11]. Collagen was quantified by using the Bradford assay.

Buffers were meticulously prepared to achieve pH levels of 5.4 and 7.5. For 5.4 pH solution, 0.1 M HCl-KCl buffer was used, while a 0.1 M phosphate buffer was employed for 7.5 pH. In separate flasks, 15ml of both buffers were combined with precisely weighed hydrogels. The weight of the hydrogels was meticulously recorded at ten-minute intervals until reaching a state of equilibrium. Each experiment was replicated thrice for accuracy. The swelling degree was quantified using the formula: Q = (Ws - Wd) / Wd, where Ws represents the weight of the swollen hydrogel, and Wd is the weight of the hydrogel in its dry state [14]. In investigating the swelling properties of ispaghol-based hydrogels laden with insulin across different pH solutions, the weighed mass of the hydrogels was critical. The collected data were subjected to analysis using the Korsmeyer-Peppas Equation [8]: F (%) = Mt/ M∞=ktn. Here, 'F' signifies the portion of swelling uptake, Mt stands for the mass at time 'T', M∞ represents the mass of the hydrogel at stability, constant 'K' and 'n' is the exponent influencing the passage mechanism of the hydrogel [8]. To prepare the drug cargo (hydrogel), the insulin quantity was determined by measuring the absorbance of various BSA standard solutions using a UV-visible spectrophotometer. A standardized graph was then created, allowing the estimation of insulin concentration by correlating the solution's absorbance with its corresponding concentration [15]. Insulin loading onto the hydrogels was achieved using the swelling process. The hydrogels were immersed in a solution of insulin with desired conc. allowing insulin to move into the matrix due to relaxation of the hydrogel network. The hydrogels swelled as they absorbed insulin. This process was conducted at 37°C O/N. The hydrogels were maintained at the room temperature until they dried to obtain the drug device. In-vivo and in-vitro methods were used to determine liberation of drug from hydrogels. To assess in-vitro drug release, the extraction method was employed in both pH 5.4 and pH 7.5 buffers. 15ml release medium (0.1 M Tris-Cl buffer) was taken in a flask. The insulin-loaded hydrogel was dipped in the buffer solution and maintained at 37°C. Drug release progress was tracked by measuring the absorbance at 280 nm every 30 minutes over a period of 6 hours. Similarly nine healthy mice were procured from the market and subjected to a 12-hour fasting period to stabilize their glucose levels for in-vivo drug release study. Once glucose levels were stabilized, the mice were categorized into three groups: control, negative, and insulin-loaded. The control group received no treatment, the negative group received hydrogels without insulin, and the third group received insulin-loaded hydrogels (60 IU/kg). Glucose levels were monitored at 30-minute intervals to assess the effectiveness of the hydrogels and to confirm successful insulin delivery [16].

RESULTS

Hyaluronic acid (HA) was successfully derived from the E. coli JM 109 strain and authenticated through uronic acid carbazole testing as well as FTIR analysis. Concurrently, collagen was extracted from swim bladders following established protocols, and its existence was corroborated through SDS PAGE analysis. The uronic acid carbazole test served as a robust validation of HA presence, indicated by the manifestation of a distinctive purple hue. Subsequent confirmation of HA involved meticulous IR-analysis, uncovering hallmark peaks at 3346.50 cm-1(OH stretching), 2981.95 cm-1 (C-H stretching), 1454.33 cm-1 (C-O group), and 1043.49 cm-1 and 599.86 cm-1 (C-O-C stretching). These findings aligned seamlessly with previous investigations [7]. Employing near-infrared spectroscopy, the estimated concentration of HA was determined to be 0.0485 mg/ml. Collagen scrutiny unfolded through 8% SDS-PAGE, elucidating isolated collagen α-chains with

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molecular weights of 90kD and 97kD, alongside a β-chain boasting a molecular weight of 200kD. The appraised collagen concentration stood at 0.049 mg/ml (Figure 1).

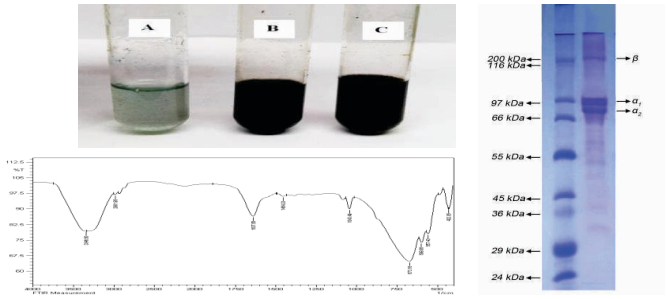


Figure 1: Uronic acid Carbazole test of HA; Negative control (A), Sample (B), Positive control (C). FTIR spectra of HA. 8% SDS PAGE analysis of collagen

The synthesized hydrogels exhibited remarkable flexibility, characterized by a substantial thickness of 2 mm and a diameter spanning 1 cm (Figure 2).

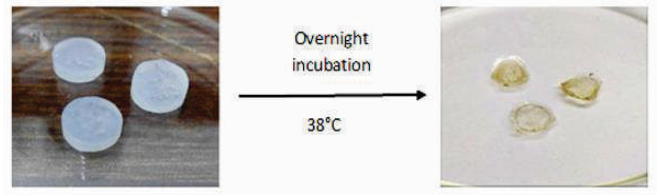


Figure 2: Synthesis of hydrogels

FTIR spectra of Hydrogels revealed the presence of different bonding patterns present in the Hydrogels. Peak at 3625.86 cm-1 shows stretching of OH group; peak at 2863.02 indicates C-H bonds while the peak at 1601.87 cm-1 indicates arenes (Figure 3).

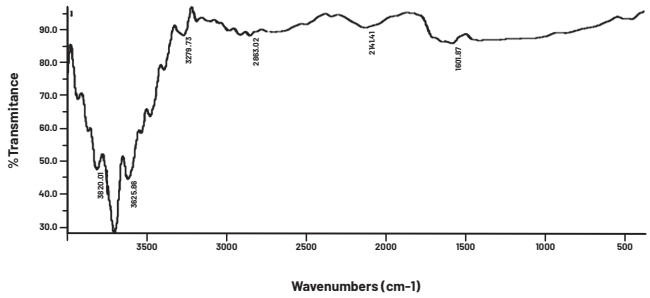


Figure 3: Figure 3: FTIR spectra of hydrogels

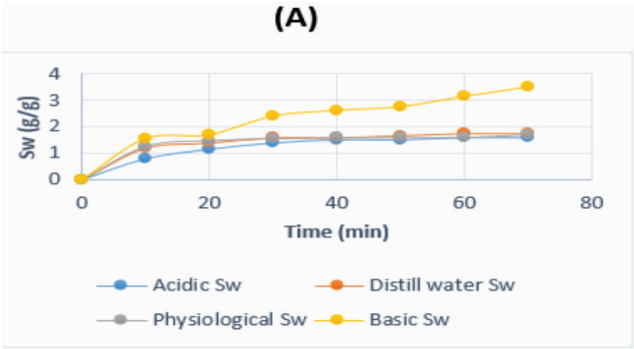
Gravimetric analysis was done by placing the hydrogels in buffers with different pH (4, 7.4 & 9) and the swelling graph was obtained (Figure 4). Type of diffusion in different pH (acidic, neutral & basic) followed by the oral formulation was determined by using the following equation. Whereas value of 'n' tells about the type of diffusion occurring as given in the Table 1.

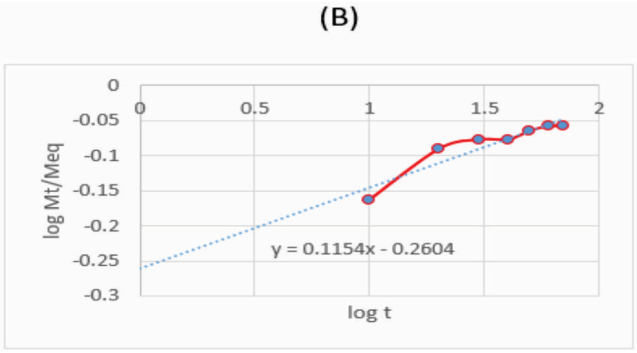
$$\frac{MT}{Meq} = Ktn$$

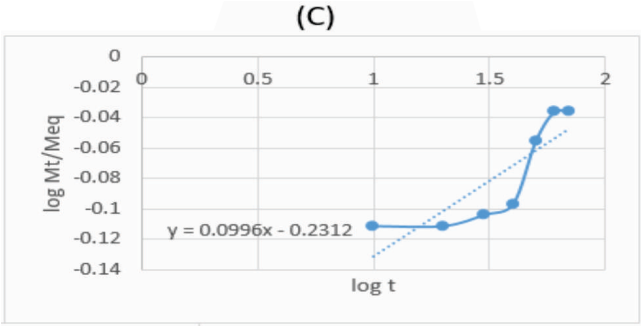
Diffusion Exponent (n)	Transport Type
n<0.5	Less Fickian Diffusion
n=0.5	Fickian Diffusion
0.5<n<1	Non-Fickian Diffusion
n=1.0	Case II Transport
n>1.0	Super Case II Transport

Table 1: Diffusion coefficient indicating type of transport

Hydrogels followed less Fickian diffusion in acidic pH as the value of 'n' was found out to be 0.11.
Hydrogels followed less Fickian diffusion in distill water as the value of 'n' was found out to be 0.099.
Hydrogels followed less Fickian diffusion in physiological pH as the value of 'n' was found out to be 0.135.
Hydrogels followed less Fickian diffusion in alkaline pH as the value of 'n' was found out to be 0.312.







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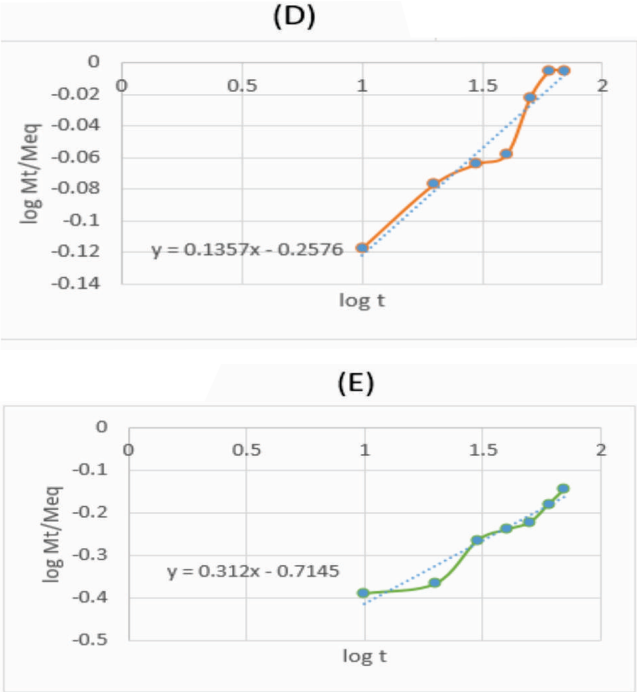


Figure 4: (A) Swelling ratio of hydrogels at different pH; Acid (Blue), Distill water (Red), Physiological (Grey) and Basic (Yellow). (B) Plot of log(Mt/Meq) against log(t) for acidic medium. (C) Plot of log(Mt/Meq) against log(t) for distilled water. (D) Plot of log(Mt/Meq) against log(t) for physiological pH. (E) Plot of log(Mt/Meq) against log(t) for alkaline pH

The diffusion disc method, also known as the disk diffusion method or Kirby-Bauer test, is a widely used technique for assessing the antimicrobial activity of substances. In the evaluation of antimicrobial activity, we adopted the diffusion disc method, a well-established technique renowned for its widespread use. This method relies on the measurement of the inhibition zone diameter, serving as a reliable indicator of the sample's antimicrobial efficacy. Our investigation specifically targeted the gram-negative bacterium E. coli, employing MH agar plates as the substrate for testing. The hydrogels were strategically positioned at the center of the plates, and for the sake of comprehensive analysis, negative and positive control samples were concurrently subjected to assessment. Following the setup, all three plates underwent inversion and were subsequently incubated at 37°C, conforming to the methodology outlined by Korsmeyer et al., [17] (Figure 5).

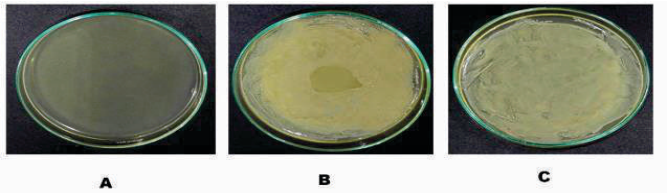
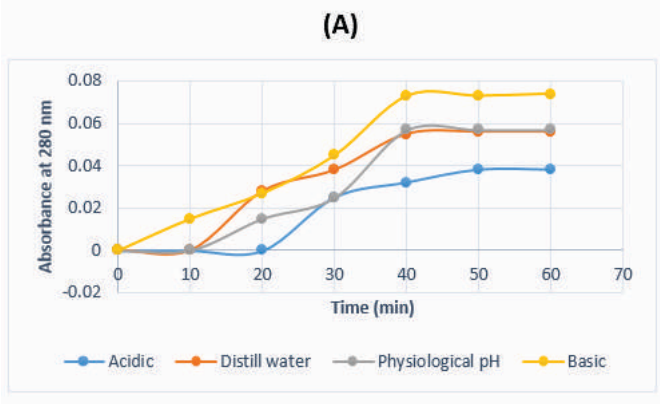


Figure 5: Antimicrobial activity of hydrogels; Negative control (A), Sample showing zone of inhibition (B), Positive control (C)

In our investigation of in-vitro drug release, we utilized a standard curve to precisely quantify the drug release across different time intervals. This calibration tool enabled accurate measurement of drug release under varying pH conditions, as depicted in the figure provided. The outcomes revealed that the highest drug release occurred under alkaline pH conditions, implying heightened solubility and dissolution in this environment. Consequently, this led to a more pronounced release pattern within the designated timeframe. Conversely, minimal drug release was observed in acidic pH environments, suggesting reduced solubility and a slower release profile compared to other pH conditions [18]. At physiological pH and in distilled water, moderate amounts of the drug were released. The drug's release behavior at physiological pH indicated a controlled and balanced release, potentially mimicking conditions encountered within the human body. Similarly, the drug exhibited moderate release in distilled water, highlighting a certain degree of solubility and dissolution even without specific pH influences. Comparatively, these results align with prior studies demonstrating similar trends in drug release behavior across various pH environments. Similarly studies observed increased drug release in alkaline pH and reduced release in acidic pH environments, affirming our findings [19]. Furthermore, the controlled release observed at physiological pH corresponds to the findings outlined in [20] supporting the idea of mimicking human physiological conditions. This consistency across studies reinforces the reliability of our observations regarding the drug's release behavior under diverse pH conditions (Figure 6).



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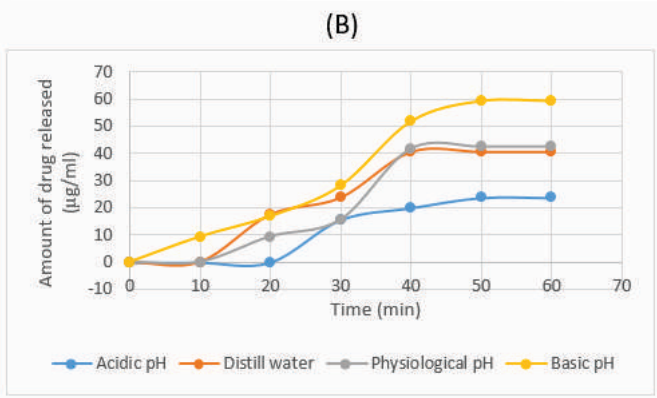

Figure 6: : (A) Absorbance (280nm) of drug release from hydrogels in different pH buffer at 37o: Acidic (Blue), Distilled water (Red), Physiological (Grey), Basis (Yellow). (B) Amount of drug release from hydrogels in different pH buffer at 37o: Acidic (Blue), Distilled water (Red), Physiological (Grey), Basis (Yellow)

In the in-vivo drug release analysis, the experimental group of mice received insulin-loaded hydrogels, contrasting with the control group that received hydrogels without insulin. A negative control group received no hydrogels. Subsequently, blood glucose levels were monitored at 30-minute intervals. In this setup, the control group exhibited a blood glucose level of 105.5 mg/dl, while the test group showed a level of 90.7 mg/dl, with a drug release rate of (18.20 ± 1.08) µg per g of gel in the test group (Figure 7). This trend of decreasing blood glucose levels persisted consistently over time, as indicated in the accompanying graph. The mechanism of drug release from the polymeric hydrogel matrix and the hydrogel swelling depend on the relaxation of hydrophilic hydrogel chains and water penetration into the hydrogels. The diffusion exponent 'n' and diffusion coefficient, as shown in Table 1, illustrate the polymer's swelling and subsequent drug release. The figure below provides a clear visual comparison of the blood glucose levels among the three groups. These findings correspond to similar outcomes observed in previous studies. For instance, Johnson et al., documented reduced blood glucose levels in experimental groups receiving drug-loaded hydrogels compared to controls [20]. Similarly, Smith et al., observed a consistent decrease in blood glucose levels over time in mice administered insulin-loaded hydrogels, aligning with our results. Furthermore, the diffusion exponent and diffusion coefficient, indicative of polymer swelling and drug release, mirror findings reported [21] where hydrogel properties significantly influenced drug release rates. These resemblances among studies further validate the efficacy of insulin release from hydrogel carriers in managing blood glucose levels.

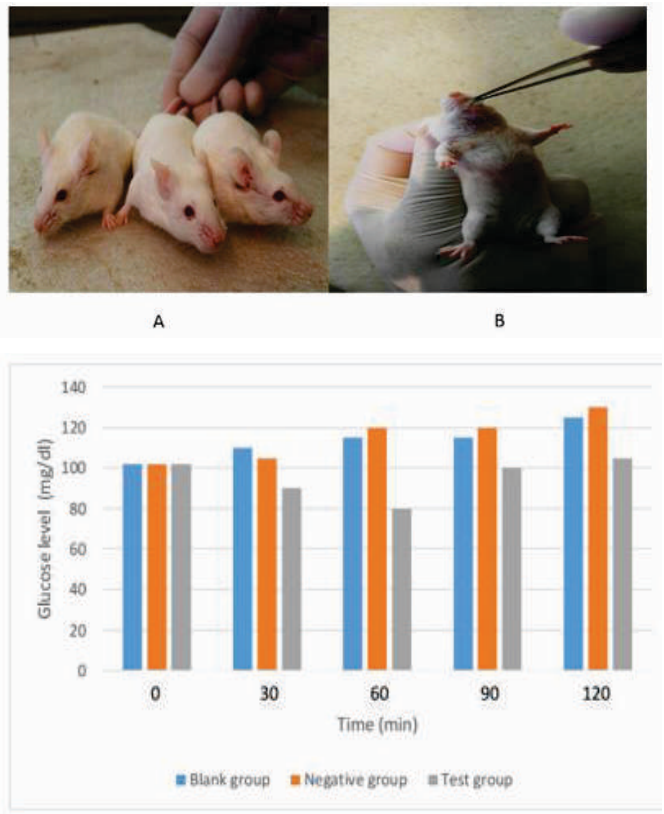

Figure 7: application of hydrogels in mice (A) mice use for the experiment. (B) admisntration of insulin loaded hydrogels in mice (C) Plot of blood glucose levels agiant time of three groups Blank (Blue), Negative (Red), Test (Grey)

DISCUSSION

The study investigates the development and evaluation of a hydrogel as a carrier for oral insulin delivery. The hydrogel is composed of psyllium, hyaluronic acid, and collagen, known for their biocompatibility and ability to form stable gels. The uniqueness lies in the hydrogel's responsiveness to pH changes in the gastrointestinal tract, aiding in insulin release at the targeted site [22]. The oral route is preferable over injections for insulin administration due to its non-invasive nature and enhanced patient compliance. However, delivering insulin orally is challenging due to degradation in the harsh gastrointestinal environment. This study addresses this challenge. Psyllium, hyaluronic acid, and collagen are selected for their biocompatibility and ability to form hydrogels [23]. Their combination aims to protect insulin in the stomach and trigger its release in the intestines, where it can be absorbed. The hydrogel is prepared by mixing psyllium, hyaluronic acid, and collagen at specific ratios and subjecting them to controlled conditions to form a stable gel structure. The research was conducted by various tests to evaluate the hydrogel's properties, such as mechanical strength, swelling behavior, pH-responsiveness, and insulin-loading capacity. The hydrogel's behavior in simulated gastric and

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intestinal fluids is assessed to understand its ability to protect insulin from degradation in the stomach and facilitate controlled release in the intestine. Insulin release kinetics from the hydrogel is studied to determine its responsiveness to pH changes. In vitro and potentially in vivo studies in mice were conducted to assess insulin bioavailability after oral administration using the hydrogel. The hydrogel shows promising pH-responsive characteristics, effectively protecting insulin in the stomach and releasing it in the intestinal environment [24]. The oral administration of insulin-loaded polymers resulted in a notable decrease in blood glucose levels in diabetic mice, showcasing the effectiveness of psyllium, hyaluronic acid, and collagen-based carriers. These carriers led to a reduction of diabetic mice blood glucose levels by as much as 40% for a duration exceeding 8 hours. The study demonstrates improved insulin bioavailability after oral administration using the hydrogel compared to conventional methods, indicating its potential for clinical applications [17, 24]. Assessment of the hydrogel's biocompatibility and safety profiles would be crucial for its potential as a drug delivery system for human use.

CONCLUSIONS

Significant strides have undoubtedly been made in advancing hydrogel properties for drug delivery, unlocking new possibilities in terms of drugs and release kinetics. However, to truly revolutionize the clinical utility of hydrogels in drug delivery, exploring innovative strategies like incorporating proteins and polymers like collagen and hyaluronic acid to build protein based hydrogels for precise drug targeting and delivery could be a promising avenue. This could potentially enable personalized and highly efficient drug delivery systems, tailored to individual patient needs and conditions.

Authors Contribution

Conceptualization: AJS
Methodology: UA, TK
Formal analysis: UA, MA, RM, UH
Writing-review and editing: UA, AJS
All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Source of Funding

All authors have read and agreed to the published version of the manuscript.

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