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HEALING POTENTIAL OF CHITOSAN PVA HYDROGELS ON EXCISED WOUND IN DIABETIC ALBINO MICE

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Abstract. Objective. *This study aimed to evaluate the efficiency of the developed chitosan/polyvinyl alcohol (CS/PVA) hydrogel crosslinked with 3-aminopropyltriethoxysilane (APTES) for its wound healing potential on diabetic wounds in mice models.* ***Methods.*** *A total of 18 Swiss albino mice were randomly assigned into a control and five treatment groups (CP0, CP50, CP100, CP200, and CP300) based on APTES crosslinker concentrations. After a 13-14 hour fast, an injection of alloxan monohydrate was used to induce type I diabetes. Mice were anesthetized, followed by the creation of a 6 mm dorsal wound using a biopsy punch. Throughout trial, wound size was measured and photographed, and blood glucose levels were monitored.* ***Results.*** *On day 15, treated groups showed complete wound healing, while the control group was in transitional stage of healing. After therapy, mice were euthanized and blood, skin, graft, kidney, and liver samples were taken for biochemical and histological investigation. Skin graft histology showed complete epithelialization and granulation in all treatment groups compared to controls. CP300 had most skin regeneration. Inflammation and necrosis were observed in the control group. Liver and kidney histological sections showed structural changes, but hydrogel induced minimal toxicity to the organs. The reported effects may have been caused by diabetes rather than hydrogels. Biochemical analysis of liver enzymes exhibited a significant ($p < 0.05$) increase in bilirubin, alkaline phosphate (ALP), alanine transaminase (ALT) and aspartate aminotransferase (AST) levels, suggesting liver dysfunction. Kidney function tests showed no significant difference in urea and creatinine concentrations.* ***Conclusion.*** *The CP300 hydrogel demonstrated an excellent healing response and is recommended as a suitable material for wound dressing.*

Key words: *APTES, chitosan/PVA hydrogels, skin grafting, type I diabetes, wound healing*

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INTRODUCTION

A major health concern, diabetes is becoming more common due to the growing global population. Chronic metabolic disease, or diabetes, affects several organ systems and is linked to several issues, including diabetic retinopathy, diabetic nephropathy, and diabetic skin ulcers [28]. Poor wound healing in people with diabetes is one of the problems that is concerning (Falanga, 2005).

Wöhler and Liebig discovered alloxan, a pyrimidine derivative they produced in 1838, that was a cytotoxic counterpart of glucose [25]. Alloxan is a primary diabetogenic compound that has a substantial impact on the field of diabetes research. Its ability to induce cytotoxicity in pancreatic beta cells is widely known, which leads to the occurrence of diabetes (Szkudelski, 2001). According to research done by Dunn et al. (1943), alloxan can cause pancreatic beta-cell necrosis in mice, which can result in diabetes [26]. Moreover, a diabetic condition that is referred to as 'chemical diabetes' or 'alloxan diabetes' occurs due to the shortage of insulin after the destruction of β-cells. This discovery increased the interest of researchers in the field of diabetic research [18, 33, 41].

According to Rodrigues et al. (2019), wound healing is based on biological process which involves vast variety of cellular elements, comprising endothelial cells fibroblasts, keratinocytes and macrophages. The primary aspect of wound healing is to repair impaired tissue, restore it to its original form and perform its function well [43]. Fibroblasts have a beneficial aspect due to their ability to deposit extracellular matrix (ECM) and are considered as an important component of this process. These cells secrete proteins such as elastin, collagen and fibronectin present in ECM [47]. These proteins offer scaffolding for cell migration and proliferation as well as controlling angiogenesis, the process of creating new blood vessels. The ECM not only provides mechanical strength to the wound site but also reduces the risk of severe scarring [13, 32].

Advanced technology has significant interest in composites, blends, and polymeric materials [24, 30, 35]. An excess number of polymers are extracted from animal proteins which includes silk, gelatin, collagen, wool, chitin, and chitosan as well as polymers extracted from carbohydrates made by fungi and bacteria. However, polysaccharides like cellulose and starch have become significant components in industrial applications [31, 44]. Chitosan has interesting characteristics like biocompatibility, antibacterial efficacy, biodegradability, and muco-adhesivity and these qualities make it good for cosmetics and especially biomedical uses [10, 16, 46]. Due to its advantageous structural characteristics like those of the extracellular matrix, it is suitable for promoting induced growth, tissue structure, and cell migration [8]. Moreover, chitosan is a biological carrier, that can move medications efficiently [42].

Hydrogels are extensively studied in industrial settings due to their categorization into chemical and physical hydrogels, as well as their potential applications in biomedicine and other beneficial properties [2, 7]. Polymer-based hydrogels which include polyacrylic acid, polyethylene glycol, polyvinyl alcohol, and others can improve tissue absorbent and are also useful for mediational uses [19]. Hydrogels also have common characteristics similar to biological tissues. It has less interfacial tension and more flexibility [12, 49]. Furthermore, these hydrogels have the ability to help tissues in regeneration processes and supply medications [27].

Polyvinyl alcohol has strong stability due to its nontoxic and water-soluble properties. However, it has quite less affinity for proteins [20]. Polyvinyl solutions are occasionally mixed with gels to create a mixture stable for medical treatments like wound healing and especially promoting wound healing in diabetic patients [5, 21]. However, wound healing in mice naturally occurs through contraction to stop this researchers use splinting rings. Excisional mouse models of diabetic wound healing show granulation and re-epithelialization, which are comparable to human wound healing processes [50]. The purpose of the present investigation is to evaluate the efficiency of chitosan/PVA hydrogel crosslinked with APTES on alloxan-induced type-I diabetics in mice models.

MATERIALS AND METHODS

Preparation of hydrogel

Hydrogels were developed through the economical solution casting procedure. 0.75 grams of CS were dissolved in 50 ml of 2% formic acid and agitated at a temperature of 60 degrees Celsius. Separately, 30 ml of distilled water was used to dissolve 0.25g of PVA that had been heated to 90 °C. The ratio of chitosan to polyvinyl alcohol utilized was 3:1. After that, both solutions were combined and blended for a period of 2 hours at 50 °C. A 50 µL solution of 3-aminopropyltriethoxysilane (APTES) in 5 ml of ethanol was added gradually to produce the blend. The resultant solution was agitated at 50 °C for further 4 hours. Subsequently, the blend was transferred into a petri dish and dried in an oven at same temperature. Once the drying process was complete, the resulting hydrogel was carefully removed and kept in polythene bags for the following analysis. Hydrogels containing APTES required concentrations (100, 200, and 300 µL) were also produced using the similar process. A hydrogel sample without any crosslinker, consisting of CS and PVA, was produced and designated as CP 0. Furthermore, CPs were labeled as CP 50, CP 100, CP 200, and CP 300, corresponding to different amounts of APTES (50, 100, 200, and 300 µL) used [4].

Animal Rearing

The Swiss albino mice used in the experiment were obtained from the Institute of Zoology, University of the Punjab. The university's animal and ethical committees gave its approval for the experiment to be carried out. Steel cages were used as animal-rearing facilities. Throughout the raising process, standard conditions were maintained, including a 12-hour light-dark cycle, a temperature of 19-21°C, and a humidity range of 45-65% [34]. The animals were provided with mineral water and commercially manufactured feed as ad libitum in the form of pellets enriched with vitamins and proteins (National Feed Lahore, Punjab, Pakistan).

The male and female albino mice, aged 8 weeks and weighing roughly 28 ± 2 g, were placed in separate cages and left there for a duration of 1-2 weeks [22. They were allowed to acclimatize. After a week, two females and one male were assigned to raise the colonies [11].

Diabetes induction

Mice were given an intraperitoneal injection of alloxan monohydrate to induce type 1 diabetes; following a 13-14-hour fast, the animals were then given a 10% glucose solution [9]. After the mice were given diabetes for 24 hours, blood samples were obtained by puncturing the tip of their tails [29]. The blood glucose level was determined using an electronic glucometer. If the blood glucose level was ≥ 250 mg/dl, diabetes has been identified. These animals with diabetes were utilized in experiments [6]. The mice's blood glucose level was noted at the start of the experiment. Mice with elevated blood glucose levels were not allowed for the experiment.

Experimental Design

Eighteen mice were randomly assigned to the six groups each comprising 3 mice. From each group, mice received intraperitoneal injections given with the combination of ketamine, xylazine and saline (400 µL, 100 µL and 1500 µL) solution for anesthesia [47]. Each mouse was administered a volume of 250 µL from the anesthetic solution. Following back preparation, mouse hair was clipped out using an electric trimmer. Draping was done using 70% ethanol [20]. With a biopsy punch, 6 mm of skin was removed from a preselected location to complete a double full-thickness excision. For 14 days, mice with wounds were consistently treated with chitosan/PVA. Different concentrations of chitosan/PVA hydrogel were given to each mouse that had a wound, such as treatment-1 received hydrogel polymer 0 µL, treatment-2 received 50 µL, treatment-3 received 100 µL, treatment-4 received 200 µL, and treatment-5 received 300 µL while the sixth group was aided as a control group with wound remained untreated. By taking pictures of the wound, the reduction in size and the re-epithelialization were measured on the 0th, 3rd, 7th, 11th, and 15th days.

Calculation Wound Surface Area

Photographs were captured of the wounded region on day 0, 3rd, 7th, 11th, and 15th. Total wound contraction was determined by the following:

$$TWC\ \% = 100 - [(Wt / Wi) \times 100]$$

Wt = Wound surface area at different time intervals; Wi = Initial wound surface area

On day 15, mice were euthanized following inhalation of 5% isoflurane. The regenerated skin grafts obtained from the wounded region were excised and prepared for histological procedure.

Histological examination

On days 7 and 14, the skin around the wound was removed from each group of mice and submerged in 10% formalin for fixation. Tissue samples were processed and embedded in paraffin after fixing. Thick sections of 3-5 µm were cut with a cryostat microtome and then allowed to stain with eosin and hematoxylin [14]. Using a light microscope, slides were examined, 10X and 40X magnification photos were taken.

Statistical analysis

SPSS (version 20.0) was employed for the statistical analysis on all of the numerical data. One-way ANOVA was undertaken using SPSS software to determine any significant differences in means. In order to compare means within each group, Tukey's test was used for post-hoc analysis. It was considered significant when the $p < 0.05$ [3].

RESULTS

Scanning Electron Microscopy

The CP0 hydrogel's morphology showed obvious depressions and asymmetrical troughs with an uneven surface. The chitosan-PVA sample that was crosslinked with 50 µL APTES showed distinctly visible pores, thin helices, and obvious, thread-like features. The CS-PVA sample that was crosslinked with 100 µL APTES exhibited a smooth surface adorned with several small pore-like features. Even though the thread-like structures had vanished, there were still a few small depressions. Furthermore, a higher quantity of micro and macropores was detected, which imparted a spongy mass-like structure to the hydrogel. In CP300, the hydrogel's porosity disappeared and took on a sponge-like appearance, causing linked helices

to form either parallel or in a circle. One of the clear causes of this hydrogel's increased swelling was its lack of pores. The SEM micrographs of the CS-PVA hydrogel that depict its surface morphology are displayed in Figure 1. The software Image J was utilized.

Evaluation of Reduction in Wound Size

After two days, the wound was checked and pictures were taken to determine the healed area shown in Figure 2. When compared to untreated, wounds treated with chitosan hydrogel showed a notable change on days 1 to 5. Hydrogel-treated wounds demonstrated 44-51% healing at day 7, indicating a significant difference across all groups. On day 7, however, CP300 displayed nearly total healing, whereas the control group had less potential for healing. On day 15, all other groups saw full recovery. Table 1 depicts the wound's reduced size.

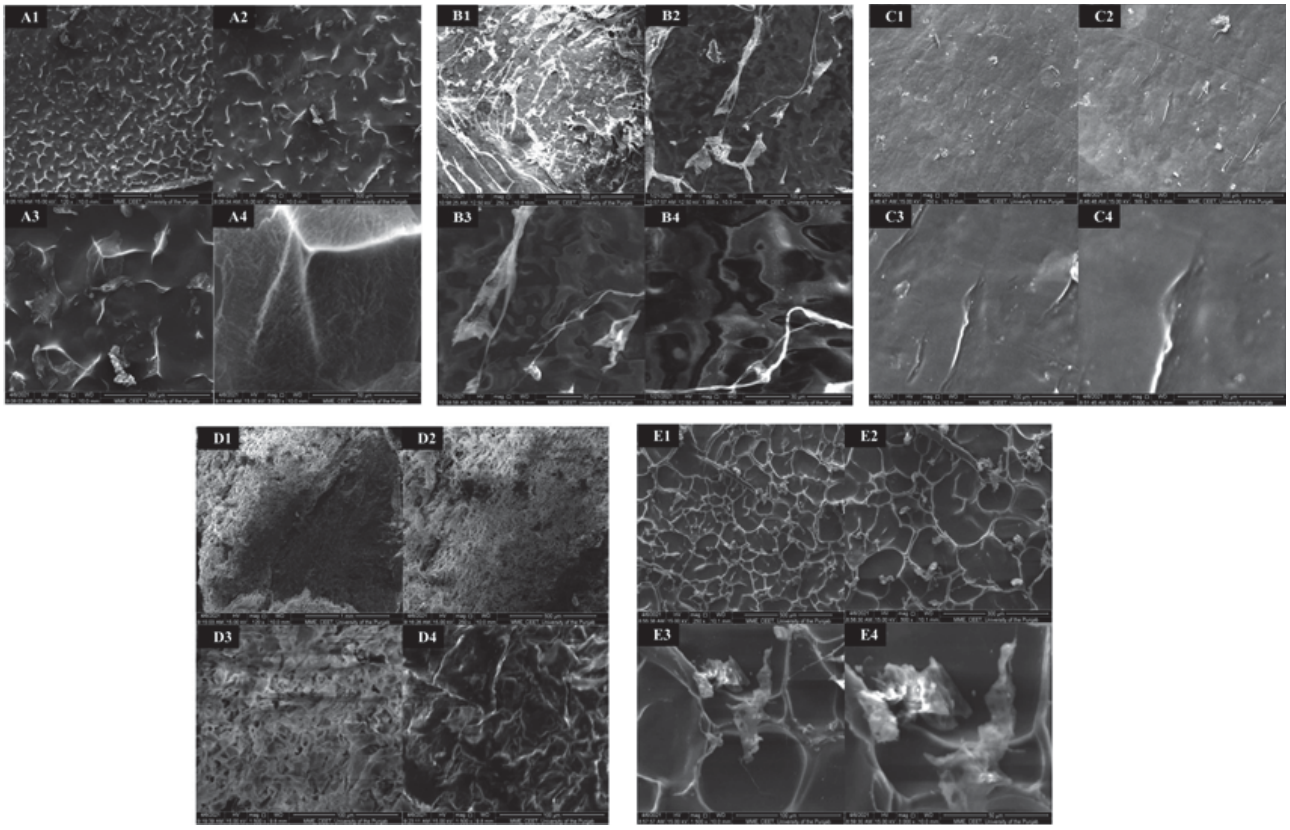

Fig. 1. Results of the morphological investigation exhibiting SEM micrographs of Chitosan-PVA hydrogels at various magnifications i.e. A = CP0, B = CP50, C= CP100, D= CP200, and E = CP300, while 1 × 250, 2 × 500, 3 × 1000 and 4 × 1500)

Table 1. Wound healing progress (wound area in mm) across different days in diabetic mice for all groups

Treatments	Day 0	Day 3	Day 7	Day 11	Day 15
Control	6.0 ± 0.0a	5.13 ± 0.12a	4.00 ± 0.008a	3.01 ± 0.01a	1.59 ± 0.04a
CP0	6.0 ± 0.0a	4.8 ± 0.11ab	3.77 ± 0.13a	2.29 ± 0.10b	0.94 ± 0.03b
CP50	6.0 ± 0.0a	4.46 ± 0.12bc	2.58 ± 0.09b	1.06 ± 0.06c	0.97 ± 0.01b
CP100	6.0 ± 0.0a	4.46 ± 0.03bc	2.30 ± 0.09b	1.01 ± 0.01c	0.84 ± 0.01c
CP200	6.0 ± 0.0a	4.10 ± 0.05c	2.19 ± 0.05b	0.95 ± 0.014c	0.42 ± 0.00d
CP300	6.0 ± 0.0a	3.30 ± 0.12d	1.36 ± 0.05c	0.65 ± 0.01d	0.00 ± 0.00e

The data were expressed in Mean ± S.E.

Small English letters a, b, c, d, and e indicated the conclusions were statistically significant ($p < 0.05$).

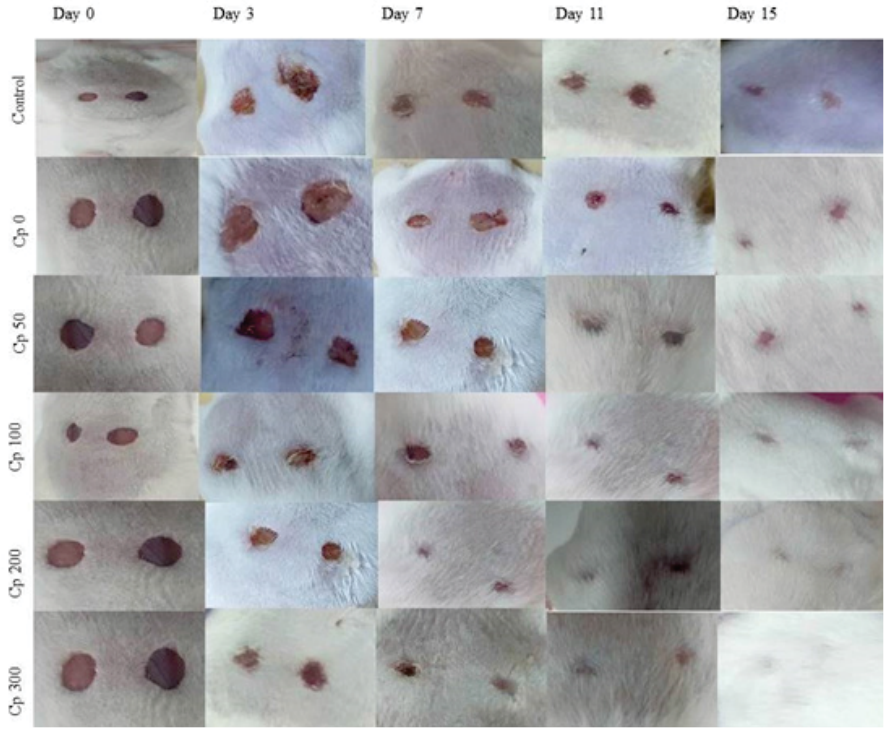

Fig. 2. Illustrate the pictures of the excised wounds in the control and hydrogel treatment groups with various concentrations i.e. CP0, CP50, CP100, CP200 and CP300

Biochemical analysis

Liver health was assessed by measuring blood levels of liver enzymes and proteins. However, kidney health was assessed by measuring levels of creatinine and urea.

Liver function tests

Table 2 demonstrated the significant impact of the chitosan/PVA polymer on the serum activity levels of bilirubin, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, and globulin. The findings exhibit that the chitosan/PVA polymer could possess hepatoprotective properties, as seen by the reduction in AST levels observed in the CP200 and CP300 groups. However, the polymer can also elicit adverse effects, as seen by the elevated ALP levels observed in the CP300 group and the reduced albumin levels observed in the CP50 group.

Renal function tests

Analyzing the levels of creatinine and urea in amniotic fluid allowed for the evaluation of renal function and maturity. The experimental results illustrated in Table 3 indicated that the renal function did not have a statistically significant effect by the chitosan/PVA polymer as determined by urea levels. There were no significant changes in urea levels reported among

Table 2. The impact of the chitosan/PVA polymer on the serum activity levels of Bilirubin, ALT, AST, ALP, Albumin, and Globulin.

Treatments	Bilirubin (mg)	ALT (U/L)	AST (U/L)	ALP (U/L)	Albumin (g/dl)	Globulin (g/dl)
Control	0.46 ± 0.03cd	124.33 ± 2.18c	120.64 ± 1.20d	401.69 ± 8.68b	3.90 ± 0.06a	2.86 ± 0.17cd
CP0	0.70 ± 0.05a	156.07 ± 0.57a	217.40 ± 0.57b	409.03 ± 2.82b	3.46 ± 0.08b	3.13 ± 0.08bc
CP50	0.60 ± 0.01b	155.15 ± 1.15a	224.12 ± 1.08a	383.86 ± 1.91c	2.86 ± 0.07c	4.16 ± 0.08a
CP100	0.56 ± 0.05b	154.03 ± 1.17a	215.52 ± 0.98b	406.73 ± 1.80b	3.60 ± 0.12b	3.15 ± 0.07bc
CP200	0.53 ± 0.02bc	142.48 ± 1.20b	134.57 ± 0.88c	373.71 ± 2.27c	2.73 ± 0.09c	3.34 ± 0.10b
CP300	0.43 ± 0.03d	126.27 ± 1.15c	118.55 ± 0.46d	436.27 ± 5.03a	3.96 ± 0.08a	2.73 ± 0.14d

The data were expressed in Mean ± S.E.

Small English letters a, b, c, d, and e indicated the conclusions were statistically significant ($p < 0.05$).

any of the treatment groups, including the control group. The CP0 group exhibited elevated creatinine levels compared to the control group, whereas no other treatment groups showed such differences.

Table 3. Effect of chitosan/PVA polymer on serum activity levels of Urea and Creatinine

Treatments	Urea	Creatinine
Control	50.66 ± .881b	0.633 ± .088b
CP0	60.33 ± .333a	0.866 ± .088a
CP50	64.00 ± 2.88a	0.733 ± .066b
C100	62.00 ± 1.00a	0.700 ± .057b
CP200	60.33 ± .881a	0.660 ± .088b
CP300	51.66 ± .881b	0.661 ± .088b

The data were expressed in Mean ± S.E.

Small English letter presented the results were statistically differ ($p < 0.05$).

Histological Analysis

Histological assessment of skin grafts

To assess histological changes during the wound healing process, tissues from the treatment group and the control group were stained at days 0, 3, 7, 11, and 15. The results are displayed in Figure 3 (a, b). On the fourth day after treatment with hydrogels, the wounds of the mice were loaded with necrotic debris and inflammatory material. In mice given chitosan/PVA, granulation tissue production was also observed, as demonstrated by eosin and hematoxylin staining. The wounded area was contracting. The control group mice's wounds exhibited proliferating blood vessels and a distorted appearance due to inflammation and necrotic debris. On day 7, the treated mice's wound area was smaller than that of the control group, which had necrotic and inflammatory material. On day 15, the regeneration of scar and epidermal tissue in the wounds of mice treated with chitosan/PVA was finished. Granulation tissue was seen and there was little space between the epidermis's wound margins. Conversely, wounds that were treated with chitosan and PVA showed the production of scar and granulation tissue as well as fibrin and acute inflammatory material in the wound pit.

Histological analysis of liver

Chitosan hydrogels appear to have some harmful effects on all treated groups, as evidenced by the his-

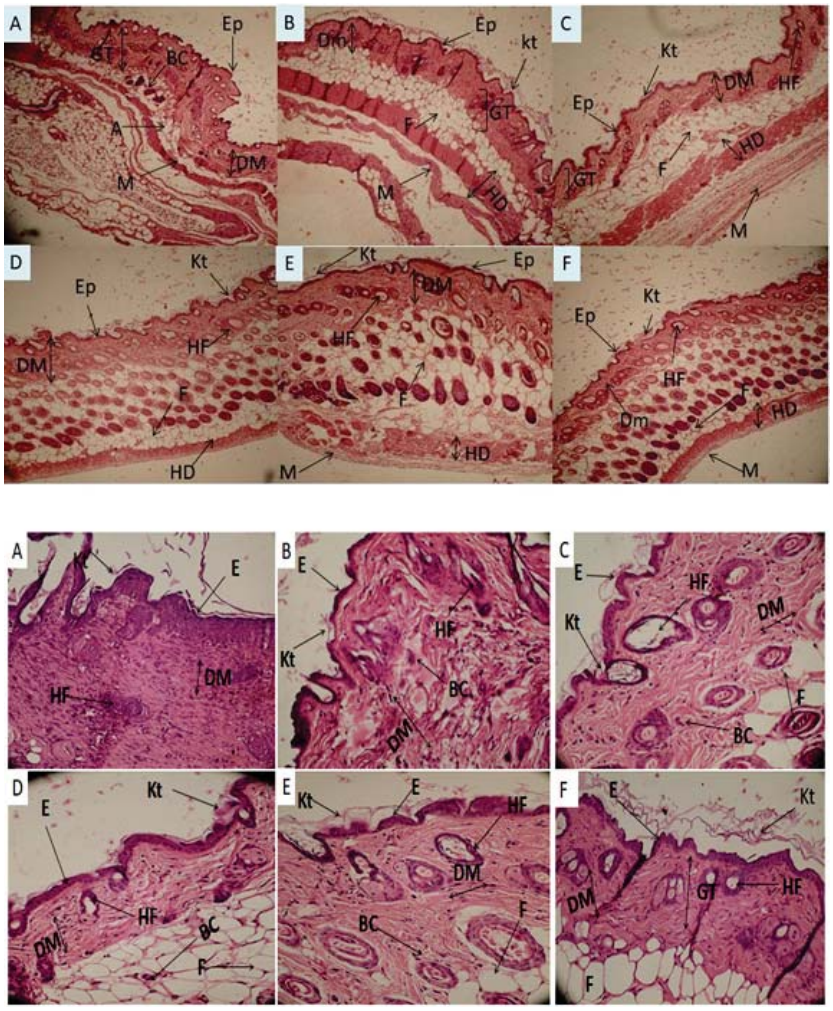

Fig. 3 (a, b). Histological section of healed skin at 15 day of control and treated groups at 10X, 40X.

Note: Control (A), CP0 (B), CP50 (C), CP100 (D), CP200 (E) and CP300 (F).

Abbreviations: Adipose (A), Blood capillaries (BC), Dermis (DM), Epidermis (E), Hypodermis (HD), Hair follicle (HF), Fat (F), Granular tissue (GT), Keratin (Kt), Muscle (M)

tological section of the treated groups exhibiting in Figure 4 (a, b) some modification in comparison to the control. There were minimal signs of blood vessel rupture, hepatocyte degeneration, and central vein congestion in higher concentrations of chitosan hydrogels. Hepatocyte infiltration into the central vein was also noted. While CP50 and CP100 showed significant results with no negative effects.

Histological analysis of kidney

All treated groups were affected by chitosan hydrogels, as evidenced by the histological section as the treatment groups exhibited in Figure 5 (a, b). There was mesangial proliferative glomerulus, glomerular atrophy, elevated bowman capsules, and renal bleeding. The histological examination of the liver and kidney revealed structural changes. However, the administration of these hydrogels resulted in lesser organ toxicity. It is worth noting that this effect may be attributed to the presence of diabetes.

DISCUSSION

The current findings demonstrate the effectiveness of PVA/chitosan hydrogels in promoting the healing of diabetic wounds in mice. In addition, it has also decreased the duration of the healing process. Chitosan-based hydrogels, which have the highest con-

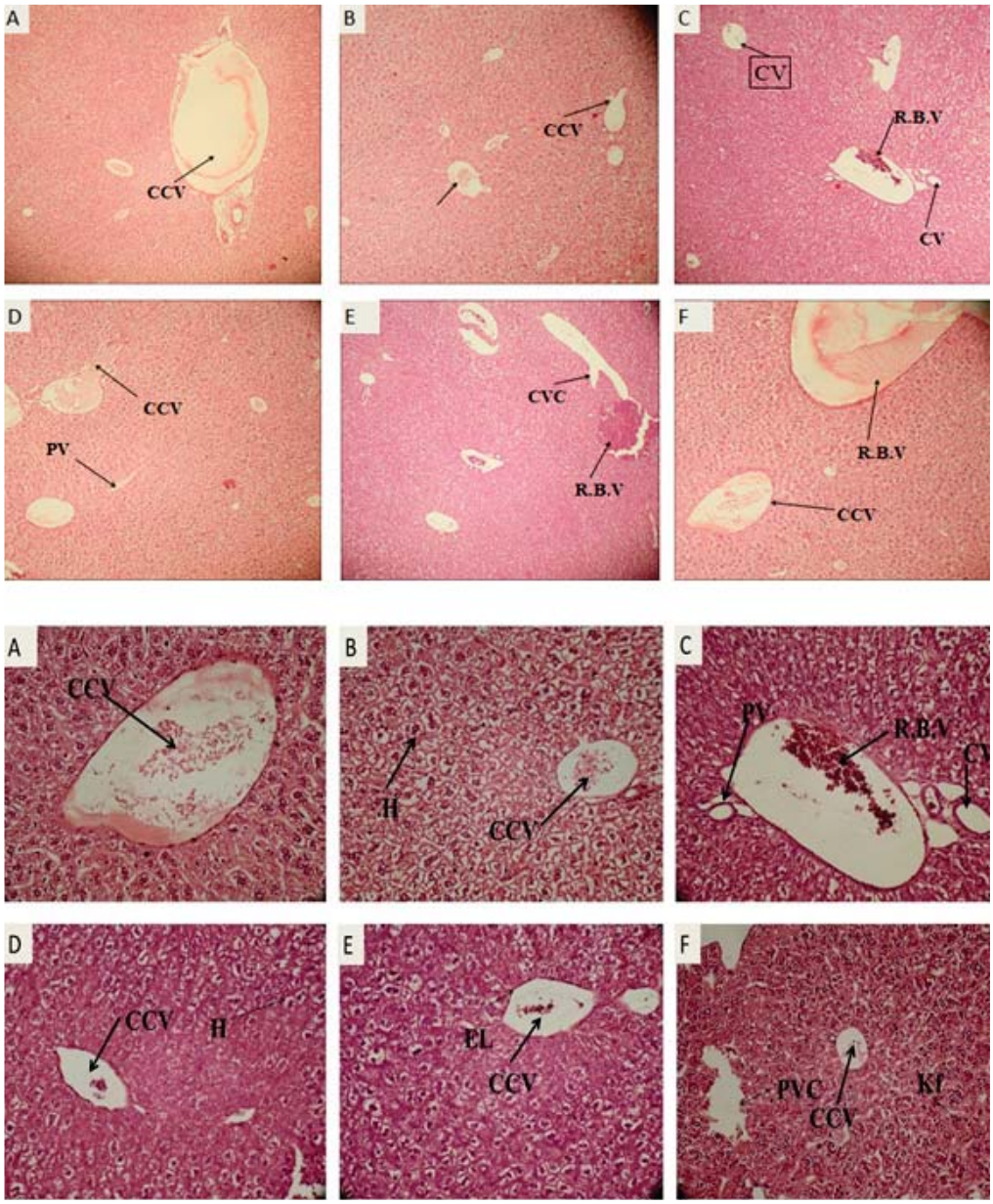

Fig. 4 (a, b). Histological section of liver at 15 day of control and treated groups at 10X, 40X

Note: Control (A), CP0 (B), CP50 (C), CP100 (D), CP200 (E) and CP300 (F)

Abbreviations: Congestion of central vein (CCV), Ruptured blood vessels (RBV), Cytoplasmic vacuolization (CV), Portal vein (PV), Epithelial layer (EL), Hepatocytes (H), and kupffer cell (Kf)

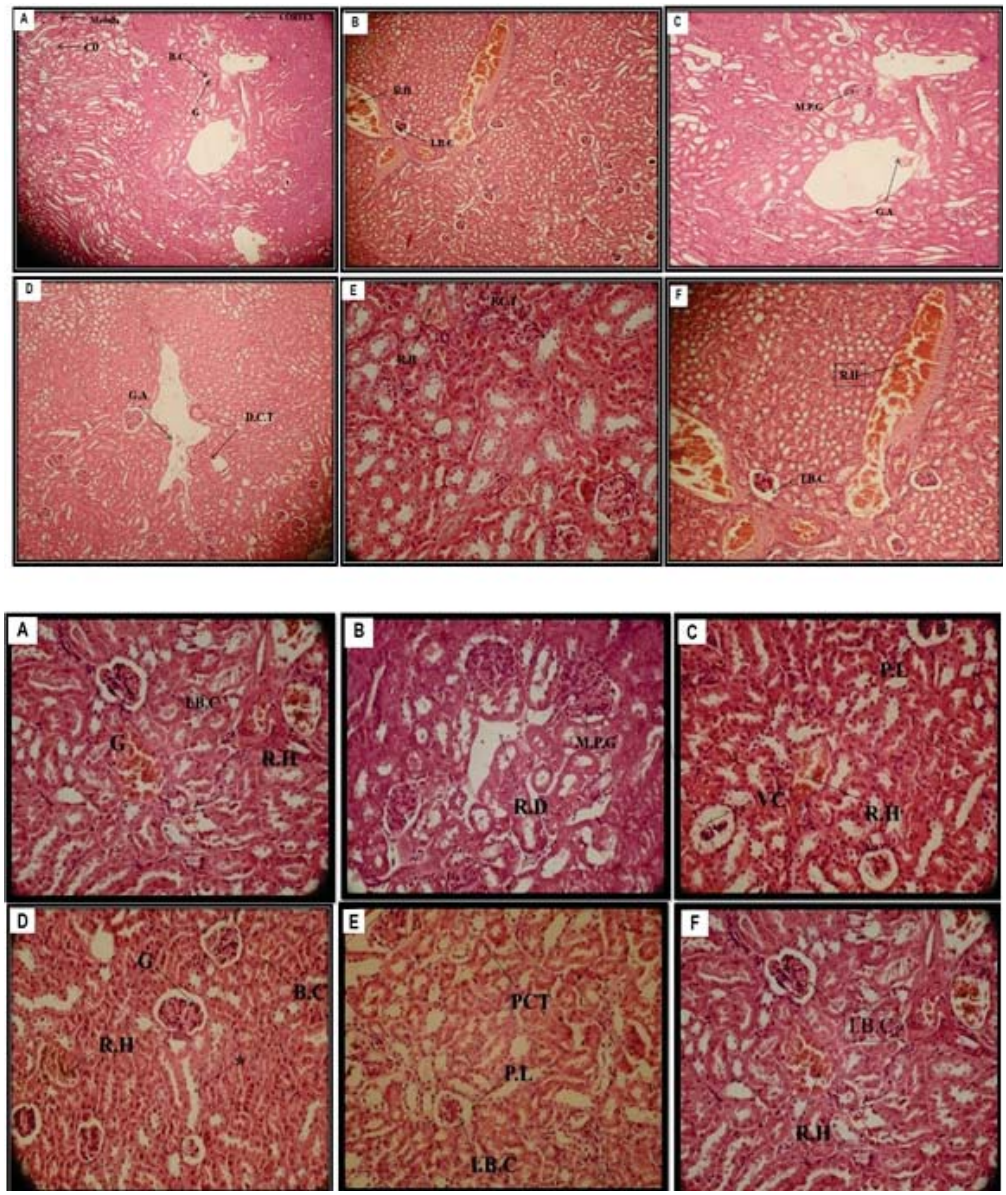

Fig. 5 (a, b). Histological section of kidney at 15 day of control and treated groups at 10X, 40X.

Note: Control (A), CP0 (B), CP50 (C), CP100 (D), CP200 (E) and CP300 (F).

Abbreviations: Bowman capsule (BC), Glomerulus (G), Increased bowman capsule (IBC), mesangial proliferative glomerulus (MPG), Renal hemorrhage (RH), Glomerulus atrophy (GA), Distal convoluted tubules (DCT), Proximal convoluted tubules (PCT), and Parietal layer (PL)

centration of 3-aminopropyltriethoxysilane (APTES), have significantly enhanced the healing potential of diabetic mouse wounds compared with groups with a lower concentration of APTES and control groups. A study also reported that high concentrations of APTES are effective for early wound healing [4].

In the present study, a composite wound dressing and a structure based on PVA/chitosan were developed by performing experiments on mice to improve diabetic patients' chronic wound healing. Chitosan is a hydrophilic polymer that aids in the repair of cutaneous injuries when mixed with hydrogels [23]. However, when cross-linked with 3-aminopropyltriethoxysilane (APTES), which is an antioxidant, this property of the compound helps accelerate the blood flow and oxygen supply to the wounded area, which ultimately speeds up the healing process [36]. Furthermore, hydrogen peroxide in chitosan shields cells from oxidative damage and free radical activity. These characteristics suggested that chitosan could be used as a scaffold and wound dressing [36].

Additionally, chitosan induced the elimination of foreign substances and kept the area moist to absorb wound fluids. Hydrogel application eliminates bacteria and aids in restorative therapy by promoting inflammation, remodeling the extracellular matrix, and regulat-

ing scar formation. Similar findings were documented by Liu et al. (2018), who examined chitosan/PVA-based wound dressings and found that they promoted tissue regeneration, eliminated exudate wounds, protected against secondary infections, improved healing process efficiency, and provided moisture to wounds. Researchers found that chitosan-based hydrogels have properties that help diabetic wounds heal faster. These properties include stopping bleeding, killing bacteria, being biocompatible, biodegradable, non-toxic, biologically active, and sticky.

On day 1, there were no differences in the wound healing rates across all groups. In contrast, the seventh day of the experiment showed a considerably higher wound closure percentage in the hydrogel treated with chitosan compared to the control group. On day 15, the group treated with hydrogel based on chitosan showed excellent capability for wound healing compared to the control group. Similar results were also reported by [4]. Our study also showed signs of regeneration in the top layer of skin, called the epidermis. However, the epithelium regeneration rate was higher in groups with higher concentrations, like CP200 and CP300, compared to control groups and groups with lower concentrations. A study by Rezvanian et al. (2021) reported similar results by investigating type I diabetes in rats and reported the material's effectiveness as a wound dressing. Vascular endothelial growth factor, histological analysis, hematology, and wound healing rate were also assessed.

Biochemical studies were conducted to examine the toxicological effects of chitosan/PVA on the kidneys and liver of mice to evaluate the toxicity. Similar results were also reported by Abed Shlaka and S Saeed (2023), in which lower concentration of polymer indicated normal appearance and had no effects, while minimal signs of adverse effects in the liver and kidneys when higher concentrations, i.e., CP300, were given in the group treated. However, the bowman capsules were increased. There was evidence of glomerulus atrophy and renal bleeding. These findings were not reported before. Histological tests for renal and liver function revealed that the hydrogel-treated groups had relatively lower levels of bilirubin, and liver enzymes such as AST, ALB, and GLB had significantly lower levels when treated with CP300 as compared to the control group. ALP and ALT have shown significantly higher levels than the control group when treated with CP300.

CONCLUSION

This study assessed the ability of chitosan/PVA hydrogel to heal diabetic wounds at various APTES crosslinker concentrations (0, 50, 100, 200, and 300 ul). Scanning electron microscopy (SEM) observations of the shape of the chitosan/PVA hydrogel revealed its porous structure. Compared to the control, wounds treated with chitosan/PVA hydrogel exhibited the most significant reduction. CP300 exhibited the highest rate of wound healing of all the treatment groups. When the chitosan/PVA hydrogel was cross-linked with APTES, it did not have many harmful effects on the liver and kidneys, as shown by biochemical parameters and histology sections. However, the reported effects may be associated with the diabetes condition rather than the hydrogel polymer itself. This finding indicates a new and hopeful avenue for further investigation, implying that chitosan/PVA hydrogel may have the ability to be used safely and effectively in diabetic patients.

This study focused on investigating the potential of chitosan-PVA hydrogels cross-linked with APTES as a possible treatment way to accelerate wound healing in diabetic albino mice with excised wounds. The study provides vital insights into the potential effectiveness of these hydrogels, presenting a novel approach to confronting the challenging aspect of impaired wound healing in diabetes conditions.

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