Original Research Article

EVALUATION OF HEALING AND MICROBIAL RESPONSES TOSURGICALWOUNDS IN ALLOXAN-INDUCEDDIABETIC RABBITS

Abstract

The wounds of diabetic patients are associated with delayed healing and microbial invasion. This study evaluated the healing and microbial responses to surgical wounds in diabetic rabbits. Sixteen (16) New Zealand White rabbitswere used in this study. They were divided into 4 groups of 4 rabbits each, namely; A, B, C and D. A is the control (non-diabetic and non-wounded), B (diabetic and non-wounded), C (wounded and non-diabetic)and D (wounded and diabetic). Three (3) cm^2 skin wounds were created in a standard aseptic condition while diabetes was induced by intravenous administration of 200 mg/kg of alloxan monohydrate.Blood samples were collected from ear vein of each rabbit on days 0,3, 7, 14, 21 and 28 post-surgery for analysis of blood glucose level. Wound samples were collected on days 3, 7, 14 and 21 for evaluation of microbial contaminants.The wounds were examined for exudation and recorded as none (0), scant (1), moderate (2) and large (3) and diameter ofwound epithelialization and contraction were determined.The blood glucose concentration ofgroups B and D from days 3 to 28 ranged between 288.00 ± 40.22 mg/dl to 358.65 ± 25.89 mg/dl),and were significantly higher ($P < 0.05$) than the correspondingvalues of groups A and C which ranged from 120.60 mg/dl ± 14.29 to 129.60 ± 8.18 mg/dl. The rate of wound contraction in the C group on day 7 (11.02 ± 2.17%) was significantly higher ($P < 0.05$) than the D group (2.23 ± 4.60%).*Staphylococcus* and *Pseudomonas* subspecies were identified in the wounds of rabbits in groups C and D. It was concluded thatalloxan-induced diabetes caused delayed wound healing while*Staphyloccocus* and *Pseudomonas species*were the predominant microorganism detected in the acute wounds of diabetic and non-diabetic rabbits.

Key words: Evaluation, wound healing, alloxan-induced diabetes, microbial infection, NZW rabbits.

Introduction

Wound healing is a fundamental, dynamic, coordinated, interdependent and overlapping cellular and immunologic responses of tissues to injury [1, 2,3]. It is essential to prevent pathogenic invasion of damaged tissues and to partially or completely reform the affected tissues [2, 3]. Experimental wounds prepared under aseptic conditions are expected to heal at a specific range of times. However, when this co-ordinated biologic process that restores tissue continuity after injury are interrupted due to some disease conditions or infections, the wound healing may delay. Such interruption may occur at any of the phases of wound healing such as angiogenesis, epithelialization; wound contraction and remodelling[4, 5].

Diabetes is a metabolic and heterogeneous disorder characterised primarily by hyperglycaemia due to deficiency of insulin or insensitivity of insulin receptors for normal processes of glucose metabolism in the body [6, 7]. The hyperglycaemia is manifested as glycosuria, hyperlipidaemia,

polyuria and polydipsia [7, 8, 9]. The disease affects man and animals worldwide[10, 11, 12].Nigeria has the greatest number of people living with diabetes in Africa [13, 14]reported a prevalence of 0.34% of diabetes mellitus among dogs attending first opinion practice in the UK.Also [15] reported a prevalence of 0.22 % of diabetic mellitus cases among dogs presented for Veterinary Care in Warri, Delta State, Nigeria.

It has been established that impaired wound healing is a notable complication of diabetes mellitus [16, 17]. Such impaired wound healing develops due to various factors associated with diabetes including slow metabolic rate, macro and microvascular complications at the edges of the wounds and peripheral neuropathy [17, 18, 19]. Diabetic wound healing in rabbits is characterised by reduced length and density of blood vessels with a greater radial diffusion distance between vessels, a less efficient network for nutrient exchange and an increase in the number of inflammatory cells and fibroblasts at few weeks post-wounding [20].

Infections have been identified as one of the major contributing factors to delayed wound healing in diabetic and normoglycemic wounds. These wound infections may include bacterial, viral and fungal infection [21]. Diabetic wounds develop more sustained and co-invasive infection that delay wound healing [22]. Consequently, wounds of diabetic patients degenerate to chronic wounds, which may defy therapeutic management [6].

Diabetes affects virtually all the stages of wound healing. The impaired healing observed in diabetic wounds correlates with decreased keratinocyte, fibroblast and immune cell migration into the wound, reduced endothelial cell angiogenesis and decreased efferocytosis [18, 23, 24]. Worthy of note is that there are numerous recent advances in therapeutic modalities targeting enhancing wound healing throughout the world. However, a thorough knowledge of association between microbial invasions of diabetic wounds and the rate of diabetic wound healing is of utmost importance in the study of wound physiology, and this poses a clinical challenge [22].[3] Proposed an ideal intervention for wound care that must involve components that act at different stages in the process of natural wound healing such as blood-clotting, anti-microbial, growth-promoting and analgesic properties.Animal models of diabetes are useful in biomedical studies because they offer the promise of new insights into human diabetes [25].This study evaluated the healing and microbial responses to surgical wounds in alloxan-induced diabetic rabbits.

Materials and Methods

Experimental animals, feeding and conditioning

Ethical clearance was obtained for this study from Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), (Reference number: ABUCAUC/2019/028). This study was carried out in the Department of Veterinary Surgery and Radiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. Sixteen adult New Zealand White (NZW) rabbits of both sexes aged between 11.6 - 12.4 months and weighing between 1.8 - 3.2 kg were used for this study. They were procured from the National Animal Production and Research Institute (NAPRI), Zaria, Kaduna State, Nigeria.

The rabbits were kept in intensive management system in separate cages and fed morning and evening daily with combined poultry grower feeds, finisher feeds (Grand Cereals Limited, Jos, Plateau State) and miller's bran that were mixed in equal proportion in order to regulate the

blood glucose level of the hyperglycaemic groups. *Daucus carota subsp. sativus* leaves was given to the rabbits twice weekly to avoid gastro-intestinal impaction and constipation while water was given *ad-libitum* throughout the experimental period.
The rabbits were conditioned to the laboratory for two weeks before the commencement of the experiment. During this period, they were administered with anthelmintic Ivermectin (Hebei, Kexing pharmaceutical, China) prophylaxis at dosage of o.4mg/kg start, and repeated after two weeks to prevent mites and other internal and external parasites. The rabbits were clinically evaluated using body condition scores, vital and haematological parameters and adjudges apparently healthy before they were certified fit for the study.

Experimental Protocols

The rabbits were divided into 4 groups (A, B, C and D) of 4 rabbits each comprising of two males and two females in each group.Group A (control): No Diabetes was induced and No Wound was created (NDNW); Group B: Diabetes was induced in this group, but No Wound was created (DNW);Group C: Wound was created, but Diabetes was Not induced (WND) andGroup D: Wound was created and Diabetes was induced (WD), respectively.

Induction of diabetes

The rabbits were first anaesthetized by intramuscular administration of 7 mg/kg xylazine hydrochloride(Bioveta a. s., Czech Republic) and 50 mg/kg ketamine hydrochloride (Laborate pharmaceutical, India). The pinna were then shaved and dabbed with xylene to dilate the marginal ear vein and 200 mg/kg of Alloxan Monohydrate(SIGMA-aldrich, UK) was administered intravenously through the marginal ear vein on two occasions with 100 mg/kg each at an interval of 72 hours [26, 27]. Blood glucose level was estimated in the blood samples collected via the ear vein using a glucometer (ACCU-CHEK $^{(R),}$ Roche, Mannheim, Germany) daily until hyperglycaemia was established and stabilized.

Wound creation

The dorsum of four rabbits each in the NDW (group C) and WD (group D) were prepared aseptically. The rabbits were anaesthetized by intramuscular injection of xylazine hydrochloride ((Bioveta a. s., Czech Republic) at 7 mg/kg and Ketamine Hydrochloride (Laborate pharmaceutical, India) at 50 mg/kg [28, 29]. A full-thickness skin wounds of 3 cm^2 (Fig.1) was created at the dorsum of each of the rabbits using a template designed from x-ray film [30]. The wounds were bandaged with sterile gauze and re-dressed only on the days of sample collections (days 0, 3, 7, 14, 21 and 28).

Blood and Microbial Sample Collections

A drop ofblood was collected from the ear vein of each of the rabbits on day 0 (before wound creation) and on days 3, 7, 14, 21 and 28 post-surgery for blood glucose evaluation.Sterile swabs were used to collect microbial samples from the wound surfaces in groups C and D on days 3, 7, 14 and 21 post-surgery for qualitative evaluation of microorganisms present in the wound.Subsequently, after the experiment, the diabetic rabbits were nursed back to their normal health using insulin to reverse diabetes.

Wound Assessment

On each day of post-surgery sample collections (days 0, 3, 7, 14, 21 and 28),the blood was placed in ACCU-check glucometer(ACCU-CHEK (R), Roche, Mannheim, Germany) and the blood glucose estimates recorded.The wounds were clinically assessed for exudation, epithelialization and contraction. The exudation was recorded as none (0), scant (1), moderate (2) and large (3) [30]. The diameter of the wounds and the epithelial region were measured using tracing papers (Fig. 2a) and a meter rule (Fig. 2b), and calculated with graph sheets (Fig. 3) to determine the epithelialization andcontraction [31, 32]. The percentage wound contraction and epithelialization were determined using the formulae described by [33].

Microbial Evaluation

The various isolation media and reagents used for this study were purchased from Oxoid®Ltd. and were prepared as described by the manufacturers [34].The swab samples were inoculated in already prepared blood agar and incubated at 37^{o}C for48 hours and clusters of microbial growth were observed. This microbial growth was further sub-cultured; A newly prepared blood agar was divided into 4 quadrants. A portion of the medium that has the microbial growth was collected using a wire loop sterilized to red hot and cooled for 10 seconds. The loop was streaked at one quadrant several times. The blood agar was turned at 90^{o}C and from the first quadrant, the second quadrant was streaked several times. The same process was repeated for the the 3rd and 4th quadrants for isolation of discrete colony. The subculture process was also carried out in MacConkey agar. The discrete colony was transferred to nutrient agar slant and incubated at 37^{o}C for 48 hours for their separation into morphological characteristics. These pure isolates were subjected to gram stains and various biochemical reactionssuch as: *Catalase test, Oxidase test, Indole test, Voges-Proaskauer (VP) Test, Methyl Red, Methyl Red, Citrate Utilization, Motility test, Urease test, TSI test, Urease test, Mannitol test, Coagulase test, Maltose test, Arginine test* [35, 36, 37].

Data Analysis

The data obtained from blood glucose estimation and wound assessment, wereexpressed as mean ± Standard Error of Mean (SEM), and subjected to statistical packages with Graph-Pad Prism version 5.03andsubsequently analysed using One Way Analysis of Variance (ANOVA) and Friedman test. Values of $P = .05$ were considered significant. The results were presented in tables and figures.

Results

The mean blood glucose level of the rabbits in group B was lowest on pre-surgery day (0), and increased sharply on post-surgery day3 (329.40 ± 61.56), and remained at high value until day 28 (273.60 ± 37.26). The mean blood glucose level of rabbits in group Dwas lowest on Pre-surgery day 0(113.80 ± 12.50), increased sharply and reached the peak on day 3 (344.70 ± 50.29) and

remained constantly high until 28 (295.20 ± 42.57). There were no significant difference ($P > 0.05$) in the mean blood glucose level of the experimental rabbits of the different groups before the induction of diabetes (day 0). Following induction of diabetes, the mean blood glucose level of groups B and D rabbits rose significantly ($P < 0.05$) and were higher than the levels in groups A and C rabbits from day 3 to 28 (Fig. 4). There was no significant difference ($P > 0.05$) between the mean blood glucose values of groups B and D; and also between the values in groups A and C rabbits.

The mean exudates value in group C was highest on day 3 (1.75 ± 0.25) while that of the value of group D was highest on day 3 (1.75 ± 0.25) and 7 (1.75 ± 0.25) before the values in both groups declined progressively to approximately 0.00 ± 0.00 on day 21 (Fig. 5). There was no significant difference ($P > 0.05$) in the exudate value observed between the two wounded (WND and WD) groups.

The degree of epithelialization**(%)** in group C rabbits was observed on day 14(29.37 ± 8.17) and 21(73.50 ± 1.37), while that of group D rabbits was on day14 (31.78 ± 5.20) and21(74.66 ± 7.01) (Figs 6 and 8). The wounds in both groups have healed completely by day 28 (Fig. 9). There was no significant difference ($P > 0.05$) between the values of percentage wound epithelialization of rabbits in group C and D throughout the period of study. However, the percentage wound epithelialization observed in group (D) rabbits on day 14 and 21 were slightly higher than the corresponding values observed in group Crabbits (Fig. 6).

The percentage wound contraction in rabbits in group C was lowest on day 3 (1.15 ± 3.36), but increased progressively to complete contraction on day 28 (100.00 ± 0.00). Also, the percentage wound contraction in group D rabbits was lowest on day 3 (1.02 ± 2.50) but increased progressively to complete contraction on day 28 (100.00 ± 0.00). The value of the percentage wound contraction observed on day 7 in group C (11.02 ± 2.17) was significantly higher ($P < 0.05$) than that observed in the D group (2.23 ± 4.60) (Fig. 7). There was complete wound contraction (100) in both group C and D rabbits on post-surgery day 28 (Fig. 9)

Four bacterial isolates namely; *Staphylococcus* species, *Pseudomonas* species, *Bacillus* species and *Corynebacterium* were identified from the wounds of the two wounded groups (C and D). However, biochemical test results confirmed the presence of only two bacterial species; *Staphylococcus* spp. and *Pseudomonas*spp (Table 1). Further biochemical tests result based on conventional biochemical identification of *Staphylococcus* isolate [37] revealed the presence of two *Staphylococcus* subspecies namely; *Staphylococcus aureus* and *Staphylococcus epidermidis* (Table 1).

Discussions

Wound healing is a cellular and immunologic responses of tissues to injury essential for repair of the injured tissues [1, 2, 3]. The wounds of diabetic patients are associated with delayed healing and microbial invasion of the injured tissues.

The observed rise in mean blood glucose level of rabbits in DNW and WD groups, above the normal mean blood glucose level of New Zealand White rabbits (150g/dl) was an indication of hyperglycaemia, a major sign of diabetes mellitus [27]. It was possible that alloxan destroyed the pancreatic β-cells in these rabbits and caused a deficiency of insulin secretion which resulted

in the metabolic derangements associated with Type-1 Diabetes Mellitus (T1DM). It could have also generated reactive oxygen species in a cyclic redox reaction with its hydroxyl radicals responsible for the selective necrosis and death of the pancreatic islet β-cells [38]. There was no significant difference ($P > 0.05$) between the blood glucose level of the rabbits in groups A and C and between groups B and D.

Scanty to moderate exudates was observed in the wounded rabbits in groups C and D. The wound exudates may contain fluid, proteins, serum, fibrin, and white blood cells to escapes into a wound defect and combine with necrotic cells and other waste materials in the wound to form wound exudates [39, 40]. It was probable that such components of exudates that facilitate healing such as fibrin, proteins, serum and water and at a therapeutic proportion dominated the exudates observed in both group C and D and contributed to the healing of the acute wound. The exudate in WD group remained at the peak up to psd 7 as agaistpsd 3 in group C probable because there was a delay in the metabolic processes that facilitate the resorption of the preparatory phases of wound healing in this WD group. Such delay could cause retention of these exudates at the peak value within 7 days duration in this group D when compared with group C. Scant to moderate wound fluids was beneficial to wound healing because it provided a moist environment for cell migration and supply of various nutrients needed for the healing [41, 42]. It has been reported that moist wounds heal two to three times faster than dry wounds [40]. The completed wound epithelialization and contraction within 28 days indicated that these defects healed by myofibroblast contraction and was in line with expected rate of experimental wound contraction in rabbits [43]. The loose skin at the rabbit back and linear edges of the square wounds favoured effective contraction, the linear traction forces of the myofibroblasts.

The mean percentage epithelialization in rabbits in group D which was slightly higher than the value recorded in group C on post-surgery day 14 indicated delayed wound healing in group D. Epithelial tissue covering was observed higher in group D probably because the contraction was delayed, due to delayed centripetal movement of the dermis and epidermis that border the wound defect to reduce the wound size [24]. In group C, the linear traction force of the myofibroblast was at a higher rate which facilitated the loose skin to cover the epithelial tissues within an interval of assessment [43]. On the other hand, the slow body metabolism associated with diabetes delayed these wound healing processes in group D rabbits thereby exposingthe epithelial tissues for a longer duration of time for assessment[18, 19].

A shorter duration of wound contraction observed in group C indicated faster granulation and epithelialization which prepared the wound bed for faster contraction in this group when compared with group D. It might also be that there was a delay in the earlier preparatory phase of wound healing such as regression and haemostasis which consequently delayed contraction from psd 3 to 14 in group D. Hyperglycaemia following diabetes caused excessive thickening of the basement membrane of blood vessels at the edges of skin wounds which resulted in poor vascular network and disorganized stages of wound healing, (18, 19].Hyperglycaemia has also been implicated in the decreased population of endothelial progenitor cells, progression of macro and microvascular complication, dysfunctional and loss of extracellular integrity and decreased efferocytosis all of which delay wound angiogenesis (19, 24].

The minimal organism isolated in this work might be due to comprehensive aseptic measures employed both in the surgery theatre, animal house and throughout the period of wound assessment. These two organisms (*Staphylococcus* and *Pseudomonas*) have always been isolated from acute, traumatic and diabetic wounds probably because they are endemic and ubiquitous in

our environment, opportunistic in nature and have high affinity in wounds [37]. It has been reported that *Staphylococcus aurous* is the most frequently isolated organisms from diabetic foot infection [44, 45. Samples from the wound of diabetic patients collected from the hospital yielded only *Staphylococcus* spp. and *E. coli* [46]. The wounds created in this research healed within twenty-one days which indicated a process of normal acute wound healing, and therefore justified the isolation of the two organisms (*Staphylococcus* and *Pseudomonas*) that have been previously reported to be common in acute wounds [47].

It was concluded that alloxan-induced diabetes causes delayed wound healing at all the phases of wound healing in NZW rabbits and*Staphyloccocus* and *Pseudomonas species* are the predominant microorganism isolated in the acute wounds of diabetic and non-diabetic NZW rabbits.It is recommended that other microbial organisms such as fungal, viral and parasitic organisms associated with wounds of diabetic animals should be evaluated.

Ethical approval:

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved byAhmadu Bello University Committee on Animal Use and Care (ABUCAUC), (Reference number: ABUCAUC/2019/028).

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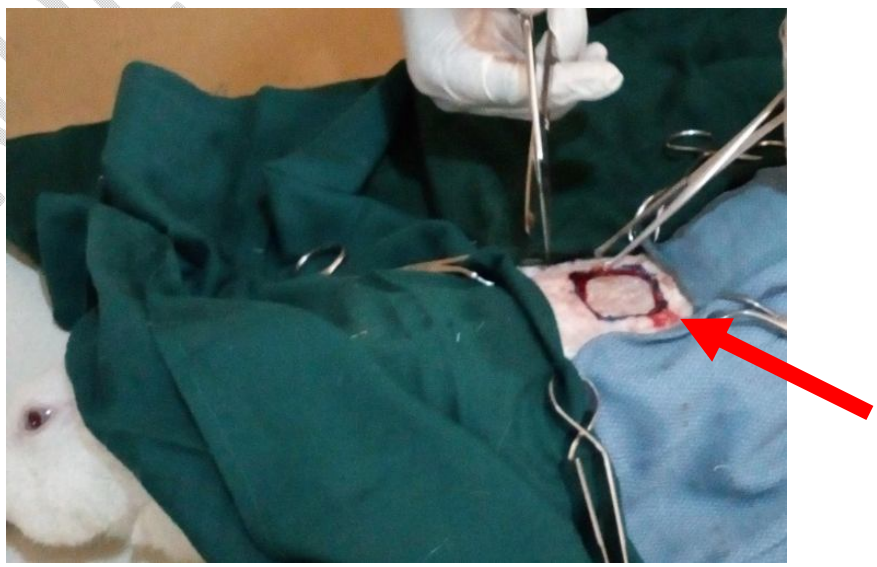

Fig. 1. The 3 cm^2 full-thickness wound (arrow) created at the aseptically draped back of the New Zealand White Rabbit.

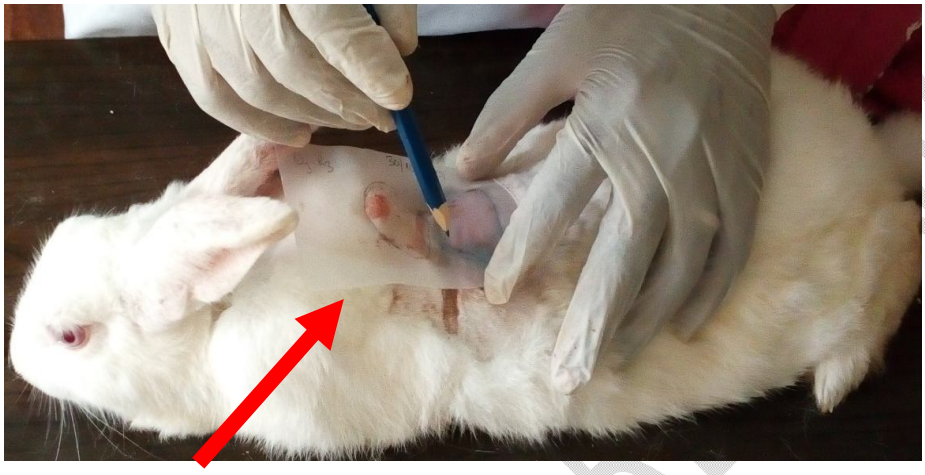

Fig. 2a.Marking of wound diameter using a transparent tracing paper (arrow).

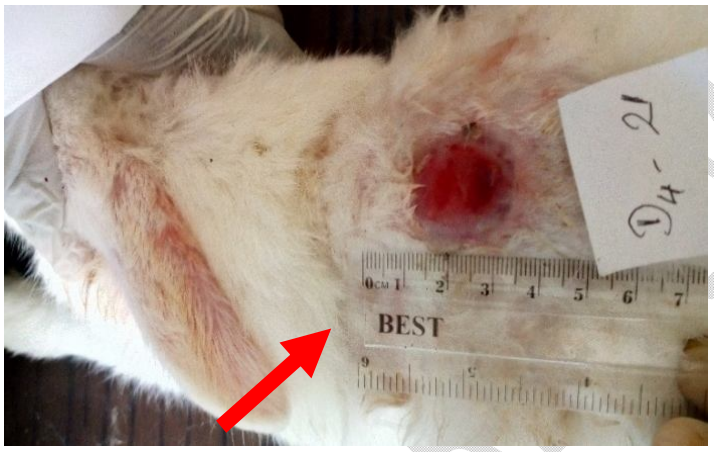

Fig. 2b Measurement of wound diameter using a meter rule (arrow).

Fig 3.Calculationof the size and epithelial tissues in the wound using a tracing paper in agraph sheet.

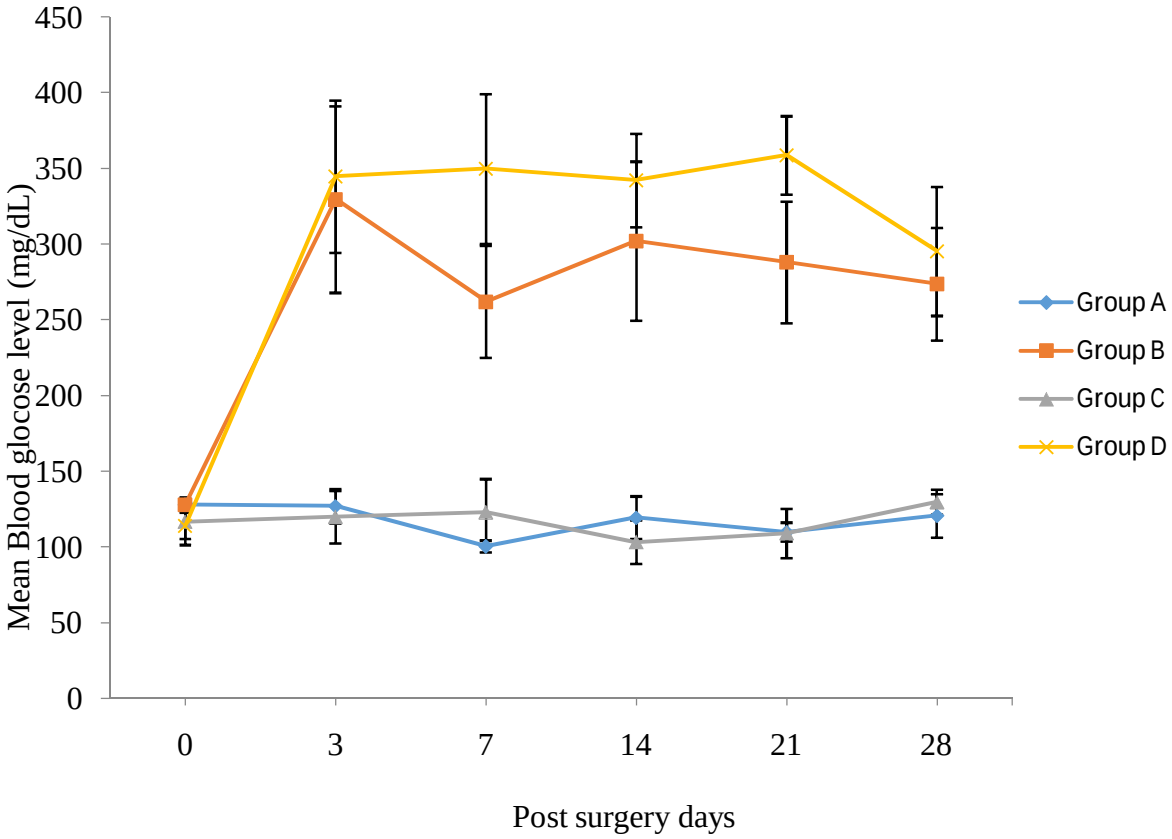

Fig. 4. The mean ± SEM blood glucose concentrationin rabbits of experimental groups (P ≤ 0.05).

Key:

Group A = Non-Diabetic and Non-Wounded (NDNW).
Group B = Diabetic and Non-Wounded (DNW).
Group C=Wounded and Non-Diabetic (WND).
Group D = Wounded and Diabetic (WD).

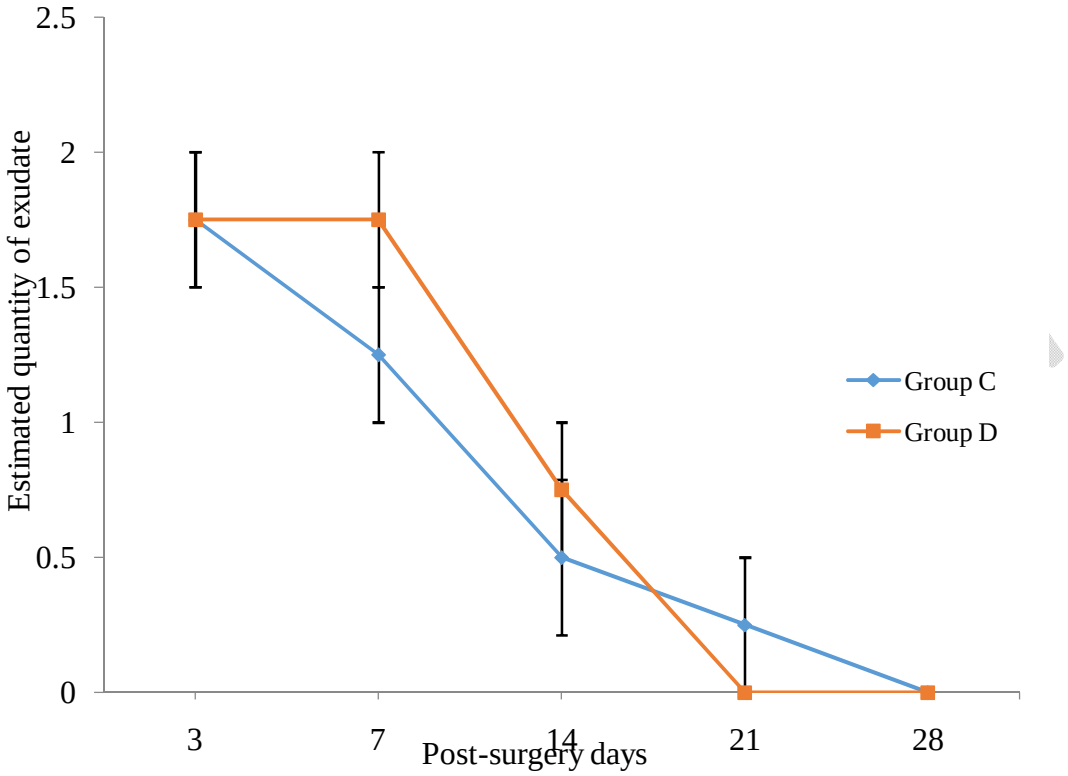

Fig. 5. The Mean ± SEM wound exudates value inc and d groups of experimental rabbits (P ≤ 0.05).

Key:

Group C=Wounded and Non-Diabetic (WND).
Group D = Wounded and Diabetic (WD).

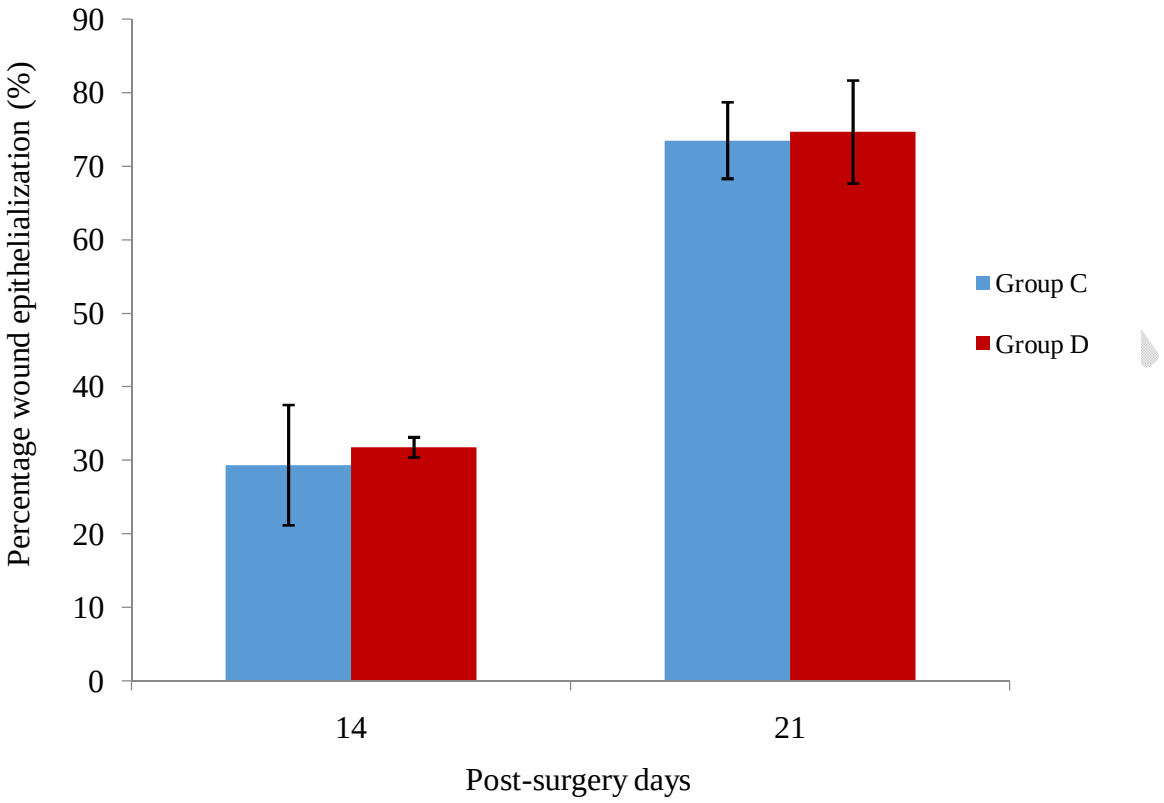

Fig. 6. The Mean ± SEM percent wound epithelialization inthe c and d groups of the experimental rabbits (P ≤ 0.05).

Key:
Group C=Wounded and Non-Diabetic (WND).
Group D = Wounded and Diabetic (WD).

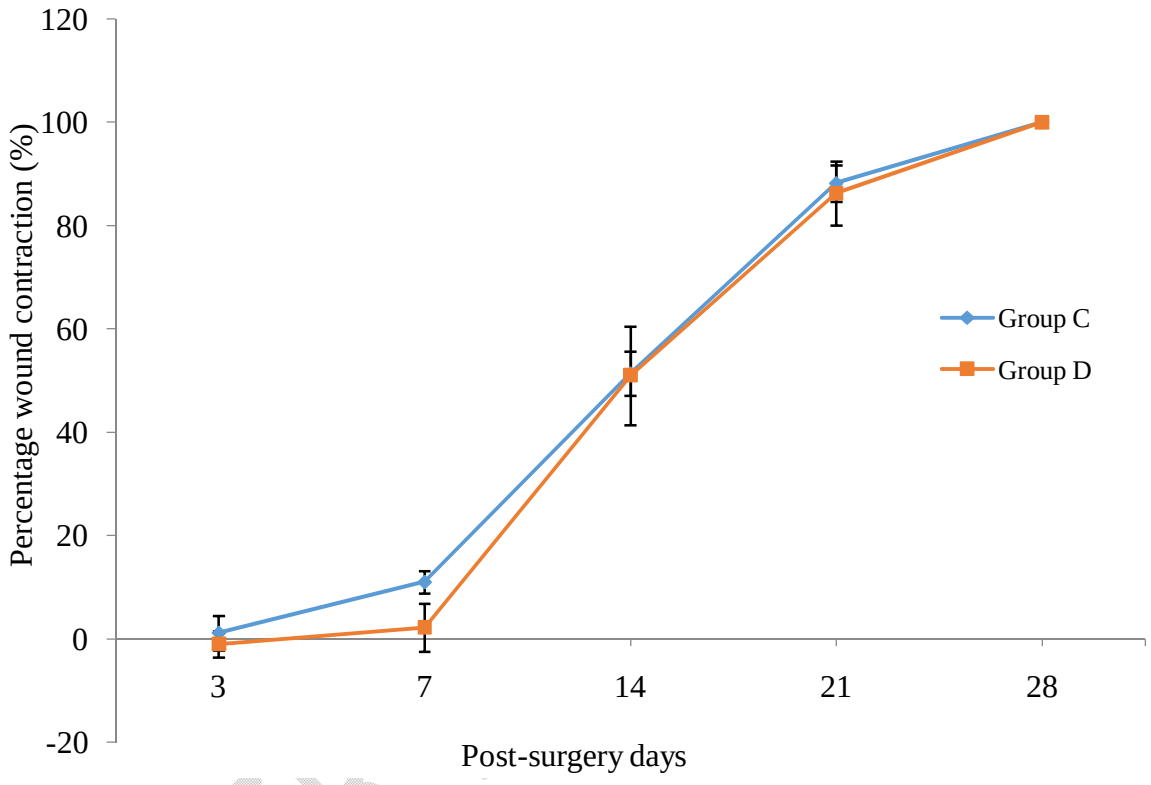

Fig. 7. The Mean ± SEM percent wound contraction inthe c and d groups of experimental rabbits (P ≤ 0.05).

Key:
Group C=Wounded and Non-Diabetic(WND).
Group D = Wounded and Diabetic (WD).

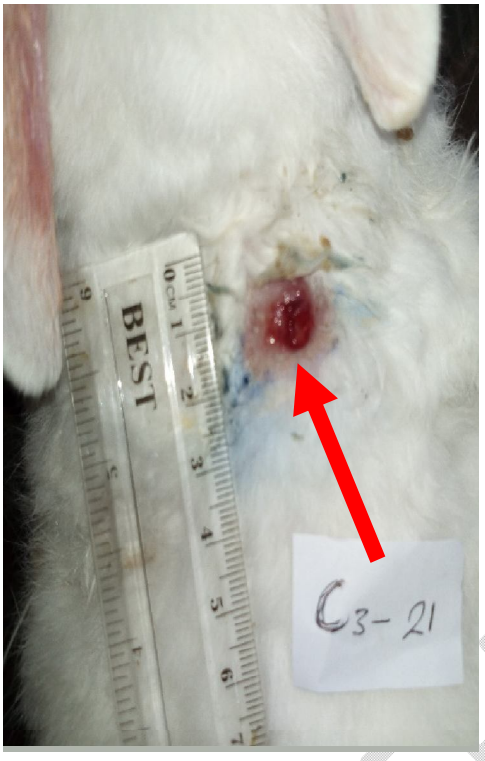

Fig. 8. The Wound of the C and D Rabbit Groups on Psd21 at the terminal Stage ofEpithelialization and Contraction (sizes of the wounds reduced more than half (arrows))

Key:

Group C=Wounded and Non-Diabetic(WND).

Group D = Wounded and Diabetic (WD)

C_3_21= Rabbit number 3 in group C on psd 21

D_4_21= Rabbit number 4 in group D on psd 21

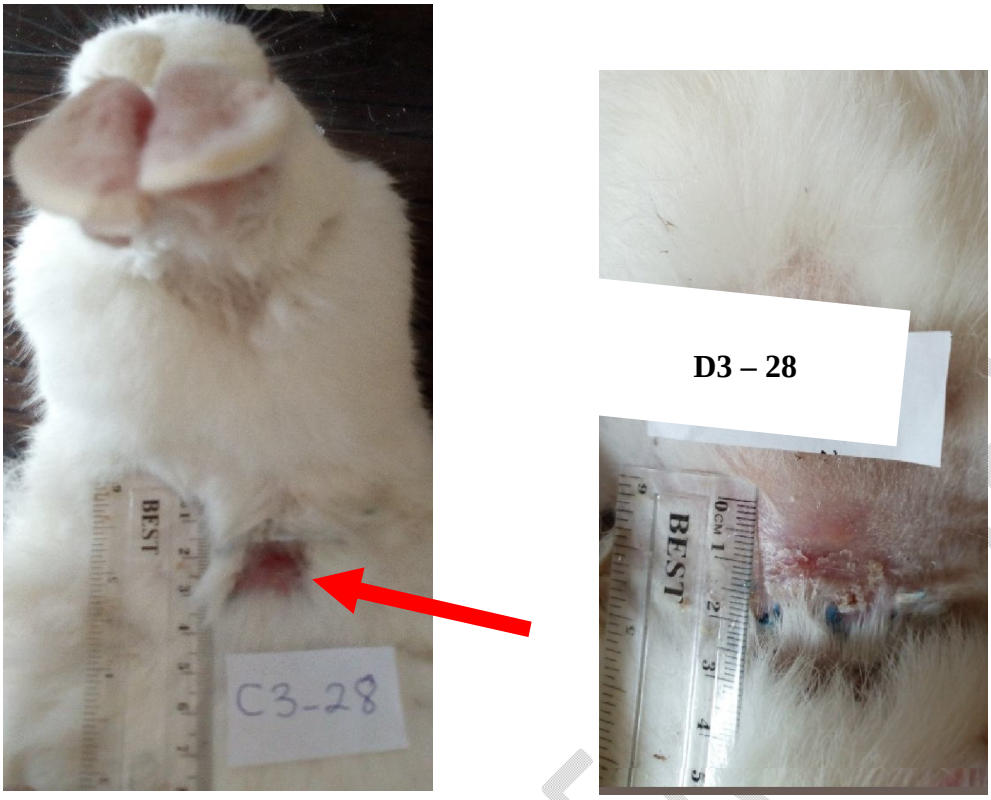

Fig. 9. The Wound of the C and D Rabbit Groups on Psd28 with Complete Contraction, and Scar Formation (arrows).

Key:

Group C=Wounded and Non-Diabetic(WND).

Group D = Wounded and Diabetic (WD).

C_3–28 = Rabbit number 3 in group C on psd 28

D_3 –28 = Rabbit number 3 in group D on psd 28

Biochemical test	***Staphylococcus aureus sub. aureus***	***Staphylococcus epidermindis***	***Pseudomonas spp***
Grams reaction	**+ cocci**	**+ cocci**	**- rod**
Catalase test	**+**	**+**	**+**
TSI test	**	**	**-**
Indole test	**	**	**-**
MR test	**	**	**-**

Table 1. The biochemical reactions of the bacterial organisms isolated and identified in the wounds of the rabbits in groups C (WND) and D (WD).

Citrate test	**	**	+
Urease test	-	+	-
Mannitol test	+	-	**
Vp. Test	-	**	-
Oxidase test	-	-	+
Coagulase test	+	+	**
Haemolysis test	+	-	+
Arginine test	+	-	**
Maltose test	+	+	-

Keys:
WND = Wounded and Non-Diabetic
WD = Wounded and Diabetic
+ = positive
- = negative
** = no test carried out
MR = methyl red.