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EFFECT OF AMLODIPINE BESYLATE FOR WOUND HEALING IN DIABETIC RATS

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ABSTRACT

Objective: Amlodipine Besylate is a long acting Calcium Channel Blocker (CCB), used in the treatment of Hypertension worldwide. The present study aims to evaluate the wound healing potential of Amlodipine Besylate in diabetic Wistar Hannover (WH) rats. **Materials and Methods**: All the selected rats were divided into 5 groups: Non-diabetic control (group I), diabetic control (group II), diabetic treated with Amlodipine Besylate 2.5 mg/Kg (group III), 5 mg/Kg (group IV) and 10 mg/Kg (group V) respectively. Rats belonging to group I and II were given the vehicle (Methyl Cellulose) only while the rats of group III, IV and V were treated with Amlodipine Besylate at the dose 2.5 mg/Kg, 5 mg/Kg and 10 mg/Kg. A full thickness excision wound of 2 cm diameter was created on the dorsal surface of all rats for measuring the wound area and percent wound contraction on day 0, 2, 5, 9, 12 and 15. Haematological, coagulation and various other biochemical parameters were also assessed on day 15. Changes in the feed consumption and body weight during week 1 and week 2 were recorded. **Results**: As compared to group I, the mean body weight of rats belonging to group II, III, IV and V was reduced significantly, while the feed consumption during week 2 was significantly enhanced. The rate of wound contraction in group V was better than all other groups. **Conclusion:** Based on results, it could be concluded that Amlodipine Besylate shows its maximum therapeutic effect at the dose of 10 mg/Kg for diabetic wound healing.

KEYWORDS: Wound healing, Diabetes, Excision, Streptozotocin (STZ).

INTRODUCTION

Diabetes Mellitus (DM) is a chronic metabolic disorder caused by the absence, insufficiency or resistance to insulin. It is characterised by derangement in carbohydrate, protein and lipid metabolism.[1] Diabetic condition leads to hyperglycaemia due to the defect in insulin secretion or insulin resistance in peripheral tissues or even both.[2] The hyperglycaemic state produce symptoms like polyurea, polydipsia, polyphagia and even weight loss.[3] Increased levels of glucose and protein glycation can cause many complications, which include cardiovascular diseases, retinopathy, neuropathy, angiopathy and nephropathy. The diabetic patients are more likely to develop nerve and blood vessel damage and often suffer from sensory loss. The symptoms of this disorder are so vast that even the small sores that develop on the feet, if get unnoticed, can develop into deeper ulcers. Further, various complication arise which necessitate amputation due to the spread of infection to the underlying tissues and bones.[4] The most traumatic symptom is of foot ulcers which require lower limb amputation and about 50% of amputations are as a result of Diabetes Mellitus only.[5]

It is estimated that more than 200 million people suffer from DM worldwide, out of which 3.4 million people died in 2004 due to this disorder.[6] Further, it is assumed that by 2030, the number of diabetic patients will increase to 439 million from 285 million (2010).[7] India has been declared as "Diabetic Capital of World" by International Diabetes Federation because 20% of the total diabetic patients in the world are found in India.[8]

Hyperglycaemia is also associated with the generation of Reactive Oxygen Species (ROS) and consequent oxidative stress.[9] It leads to several diabetic complications like wounds, ulcers, neuropathy, nephropathy and sensory loss which have been recognised as a major cause of prolonged healing in diabetic patients.[10] Many drugs are available in market which reduces the peripheral vascular resistance, blood viscosity and leads to local or even systemic vasodilatation. It has been observed that CCBs increases skin tensile strength, enhance wound contraction rate and could also partially reverse steroid suppressed healing. CCBs are known to cause vasodilatation which increase blood supply to the wounded region. Hence these CCBs could be safely used in patients undergoing surgery as they do not affect healing adversely. This pro-healing action could be utilized favourably, especially if the patient is on some known suppressor of wound healing like corticosteroid and anti malignant agents.[11]

MATERIALS AND METHODS

1. Animals

A total number of 50 WH rats (Nomenclature: HanTac: WH, Taconic, USA) weighing 150-250 g of about 5-8 weeks old were received from Animal Breeding and Housing Facility (ABHF) of Ranbaxy Research Laboratories (RRL), Gurgaon. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) of Ranbaxy Research Laboratories (Protocol No. 27/2011) as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Rats (CPCSEA), India.

2. Environment and Housing

All the rats were housed in individual cages under standard conditions of temperature 18-26^{0}C and relative humidity 30-70% respectively. During acclimatization period of 5 days, a maximum number of 5 rats were housed in one cage while in experimental period, each rat was housed individually in separate cage provided with wire mesh platform grills to avoid contamination of wounds with the bedding material. Rats were provided with autoclaved standard laboratory animal diet (Harlan Rodent Feed, Manufactured by: Harlan Netherlands B.V. Kreuzelweg 53, 5961 NM Horst (L), Netherlands) *ad libitum* during acclimatization as well as in experimental period and free access to purified water.

3. Experimental Procedures

3.1 Pre-Randomization

On the last day of acclimatization; all the rats based on their body weight were randomly divided into two groups of 10 and 40, such that the group mean body weight did not differ statistically. The body weight variation of the rat selected for the study did not exceed ± 20% of the mean body weight for each group.

3.2 Induction of Diabetes Mellitus

Diabetes was induced in 40 rats by single i.p. injection of Streptozotocin (STZ, Batch No: 031M1287V, S0130-1G, manufactured by Sigma Aldrich U.S.A, dose 50 mg/Kg) solution on day 1 i.e. a day after pre-randomization. STZ solution was prepared by dissolving the STZ powder in freshly prepared Citrate Buffer solution (0.5 M, pH 4.5). Considering the dose volume of 10 mL/Kg for an i.p. injection, the STZ solution with a concentration of 5 mg/mL of Citrate Buffer solution was prepared to achieve a dose of 50 mg/Kg body weight.[12] All 40 rats were given an i.p. injection of STZ within 15-20 minutes of its preparation due to very limited stability of the STZ. On day 7 of STZ injection, blood samples were taken to measure

the blood glucose level. Rats showing blood glucose value ≥ 300 mg/dL were only selected for randomization.

3.3 Study Design

An outline of the study design is presented in Table 1.

Table 1: An outline of study design.

Groups	Treatment	Animal No.
I	Non Diabetic Control	1-6
II	Diabetic Control	7-12
III	Diabetic + Amlodipine Besylate at the dose of 2.5 mg/Kg	13-18
IV	Diabetic + Amlodipine Besylate at the dose of 5 mg/Kg	19-24
V	Diabetic + Amlodipine Besylate at the dose of 10 mg/Kg	25-30

3.4 Wound Creation

On the next day of randomization and grouping, all the rats belonging to groups I, II, III, IV and V were anaesthetized by an i.p. injection of a mixture of Xylazine (Xyalxin, Indian Immunological Limited, dose10 mg/Kg) and Ketamine (Aneket, Neon Pharmacy, dose 75 mg/Kg). In fully anesthetized rats, the dorsal abdominal region was closely shaved with hair clipper using blade Number 10 and 40 and excess loose hairs if any, were brushed off. Then, the shaven area was cleaned by using Povidone iodine ointment (10% w/w).

For creating excision wounds, an area of 2 cm diameter was initially marked by putting an ink stamp and then a full thickness excision wound was created on the previously shaven area with a pair of sterile pointed scissors. The day of wound creation was considered as day 0 for assessing the wound parameters.

3.5 Treatment with Amlodipine Besylate

An active pharmaceutical ingredient (API) of Amlodipine Besylate (Batch No: 1943973 manufactured by Ranbaxy) was dissolved in 0.5 %w/v Methyl Cellulose for oral gavage administration to groups III, IV and V at the dose of 2.5, 5 and 10 mg/Kg body weight. The drug formulations of different concentrations were prepared just prior to daily administration by considering the dose volume 10 mL/Kg of the body weight. The dose volume to be administered was calculated based on the most recent body weight of the rats.

The test drug (Amlodipine Besylate) and vehicle (Methyl Cellulose 0.5% w/v) were administered by oral (gavage) route using disposable syringe (Dispovan, India) and rat

intubation cannula (16" gauge) once daily for 14 consecutive days. The vehicle, 0.5%w/v Methyl Cellulose (1% w/v in MilliQ water) was prepared well in advance and stored at 2–8^{o}C. The dose of Amlodipine Besylate was chosen on the basis of recommended human therapeutic dose.

3.6 Observations

3.6.1 Clinical Sign and Mortality

All the rats were observed for clinical signs once daily between 2 to 4 hours post dosing. Mortality and morbidity was recorded twice daily once before dosing and once in the evening.

3.6.2 Body Weight and Feed Consumption

Body weight and feed consumption was recorded on Day 1, 8 and 14 using Electronic Weighing Balance (Sartorius, Germany).

3.6.3 Assessment of Wound Area and Percent Wound Contraction

Serial standard 2D photographs of each rat were taken on day 0, 2, 5, 9, 12 and 15 to assess the wound closure and contraction. A digital camera (Canon DSLR-550D attached with 18-55mm Canon lens) was statically mounted on a tripod stand (Slik F133) facing down approximately 24 cm above the rat, permitting direct comparison of wounds between individual rats. The rat was placed on a specially designed cardboard platform affixed with horizontal and vertical rulers and grids created of 2 cm x 2 cm size. The rat was placed flat on its belly directly under the camera, front legs and neck slightly stretched out (i.e. wound bearing area) visible on the display. For each photographic session; rats were briefly anaesthetized with Isoflurane and a paper tag was kept to indicate the rat number.

Digital images were processed using the LEICA QWIN Image Processing and Analysis Software (Leica Microsystems, Germany), which is having Measure Interactive option that can displays the manual results for the measured object. All the photographs from each photographic session were subjected to analysis and measurements, after initial calibration of the software for horizontal, vertical and diagonal scale with the blank image of cardboard platform. All excision wounds were measured for their area and the data generated from each rat was used to calculate the average area and percent wound contraction on day 0, 2, 5, 9, 12 and 15 with respect to day 0.

$$\text{Wound contraction (\%) for day x} = \frac{\text{Day 0 – Day X}}{\text{Day 0}} \times 100$$

3.6.4 Clinical Pathology Investigations

The blood samples for clinical pathology investigations were collected from overnight (12-16 hours) fasting rats on day 15 by retro-orbital plexus route under Isoflurane anaesthesia Approximately 0.5 mL of blood was collected in each tube containing EDTA-K3 (1 mg/mL or 0.5 mL of blood) and Tri-Sodium Citrate for analysis of haematological and coagulation parameters respectively. Approximately 1.5 mL of blood was collected in tubes containing heparin [10 µL if heparin/mL of blood (Heparin: 5000 IU/mL)] for analysis of clinical chemistry parameters. For assessment of clinical chemistry and coagulation parameters, the plasma was separated from blood samples by centrifugation at 3000 rpm for 15 minutes at 2-8^{o} C.

3.6.5 Haematological Analysis

The haematological parameters were estimated using Advia 120 hematology analyzer (Germany).

3.6.6 Coagulation Analysis

The determination of coagulation parameters like Prothrombin time (PT) and Activated Partial Thromboplastin Time (APTT) were performed using Sysmex-560 (Japan) Automated blood coagulation analyzer.

3.6.7 Clinical Chemistry Analysis

The clinical parameters like concentration of Chloride (Cl^-), Creatinine, Glucose, Potassium ions, Sodium ions (Na^+) and Alanine Aminotransferase (ALT) were estimated by the respective methods with Automatic Clinical Chemistry Analyzer, Dimension R_xL, Dade Behring, USA.

3.6.8 Statistical Analysis

All the parameters were analyzed statistically, like body weight and percent change in body weight with respect to day 1, wound area measurements and per cent wound contraction with respect to day 0 and clinical laboratory investigations. The number of observations (N), mean value of each group and standard deviations (%), where applicable were calculated.

The normality of the data was examined first using D'Agostino and Pearson omnibus normality test. If data showed normal distribution, it was tested for heterogeneity of variances. If the variances were found homogeneous, one way ANOVA taking dose group (group III, IV and V), as the factor was performed. Otherwise series of transformations (Logarithm, Square-root and Inverse root, in sequential order) were applied to make the variances homogeneous. Dunnett's test was applied to compare the dose groups over the control one. Further, all the parameters including the wound area measurements and % wound contraction for group III, IV and V were also compared with group II (Diabetic Control) for statistical significance if any.

RESULTS

4.1 Clinical Signs and Mortality

Clinical signs due to Diabetes Mellitus, like excessive urination, drinking and eating were observed. Apart from this, there were formation of scabs and escher associated with injuries observed in all the rats either diabetic or non diabetic.

4.2 Body Weight and Percent change in Body Weight

There was a significant weight loss observed in the body weight of rats belonging to group II, III and IV when compared with non diabetic control (group I) rats but no such significant weight loss was observed in group V rats. The summary of body weight and percent change in body weight is presented in Table 2.

Table 2: Summary of Body Weight (g) and % Change in Body Weight with respect to day 1.

Group	Body Weight (g) (Mean ± S.D.)			% Change in Body Weight	
	Day 1	Day 7	Day 14	Day 7	Day 14
I	243.6 ±10.7	267.8±11.06	285.0±11.86	9.98±3.10	17.07±4.59
II	233.8±3.36	(245.5±8.25)*	(249.6±11.76)*	4.95±2.56	(6.70±3.82)*
III	229.0±18.06	(248.0±9.94)*	(261.8±9.20)*	8.917±10.48	14.82±7.73
IV	234.7±6.589	(243.6±11.50)*	(254.7±18.13)*	3.833±4.797	(8.52±6.57)*
V	236.5±5.368	255.1±9.807	267.7±13.84	7.883±2.551	13.18±4.727

Values are Mean ± Standard Deviation *: $p<0.05$

4.3 Feed Consumption

The mean feed consumption per rat per day for week 1 did not reveal any significant difference within groups, but in week 2 the mean feed consumption in group II, III, IV and V

has increased significantly when compared with non diabetic control (group I). However, the mean feed consumption within different treatment groups (group III, IV and V) and diabetic control (group II) did not differ either in week 1 or 2. The comparison of feed consumption within different groups is shown in Table 3.

Table 3: Summary of Feed Consumption (g/rat/day).

Group	Dose (mg/Kg body weight)	Week 1	Week 2
I	0	21.63±8.372	18.60±4.048
II	0	26.06±0.6341	(28.60±3.720)*
III	2.5	24.77±0.5422	(30.43±4.104)*
IV	5	26.48±0.5548	(30.66±4.227)*
V	10	24.99±1.098	(30.77±2.417)*

Values are Mean ± Standard Deviation

*: $p<0.05$

4.4 Wound Area Measurement and Percent Wound Contraction

The wound area and percent wound contraction measured on day 0, 2, 5, 9, 12 and 15 did not reveal any statistically significant difference within different treatment groups (group III, IV and V) and when compared with the non diabetic control (group I) and diabetic control (group II). However, the rate of percent wound contraction in non diabetic control (group I) and in diabetic rats treated with Amlodipine Besylate at the dose of 10 mg/Kg (group V) was better than all other groups (group II, III and IV) indicating faster closure of wounds in these groups. The rate of wound contraction in diabetic control group (group II) was lowest among all the groups followed by diabetic rats treated with Amlodipine Besylate at the dose of 2.5 mg/Kg (group III). The summary of mean wound area and % wound contraction is presented in Table 4 and 5. The progression of excision wound closure on various days has been shown in Figure 1-10.

Table 4: Summary of Wound Area Measurement.

Group	Wound Area measurement (in mm^2) on different days					
	0	2	5	9	12	15
I	194.9±26.4	187.3±31.3	130.0±21.0	52.6±14.0	25.7±7.7	8.5±4.2
II	185.6±14.8	182.7±25.0	132.1±17.2	78.2±21.1	41.9±11.9	26.1±7.9
III	183.9±16.5	191.0±8.5	123.7±12.0	67.3±14.4	29.1±16.1	16.1±16.1
IV	175.5±16.0	185.4±13.3	131.9±10.0	58.4±14.4	28.9±6.8	9.9±2.8
V	185.0±14.6	196.9±12.1	134.2±21.4	66.28±16.2	25.5±9.5	7.2±3.9

Values are Mean ± Standard Deviation

*: $p<0.05$

Table 5: Summary of % Wound Contraction.

Group	Dose (mg/Kg)	% Wound Contraction with respect to day 0 on different days				
		2	5	9	12	15
I	0	3.65±11.4	32.91±9.65	72.82±7.44	86.59±4.67	95.41±2.73
II	0	1.46±11.66	28.64±9.11	57.68±11.16	77.46±6.96	86.03±4.39
III	2.5	-4.45±8.69	32.68±4.42	63.10±9.16	84.14±8.98	91.21±8.93
IV	5	-6.13±8.50	24.44±7.65	67.04±6.11	83.41±4.17	94.34±1.64
V	10	-6.78±7.56	27.51±10.02	64.31±8.11	85.95±5.86	96.00±2.38

Values are Mean ± Standard Deviation

*: $p<0.05$

FIGURES

GROSS PHOTOGRAPHY OF WOUNDS ON DAY 0

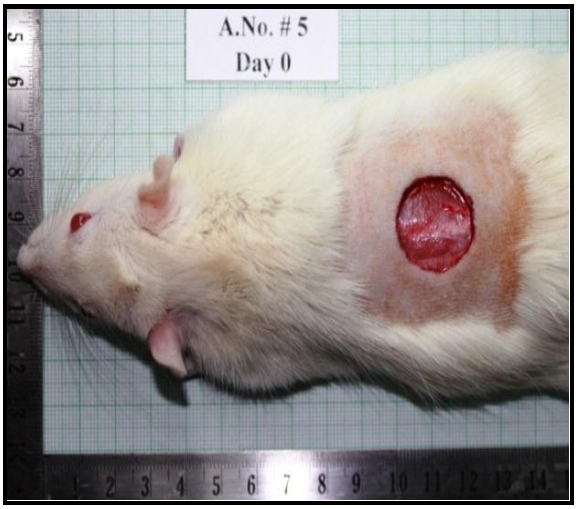


Figure 1

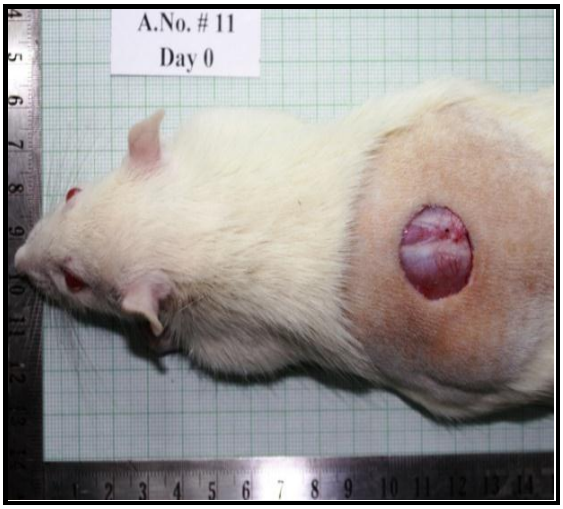


Figure 2

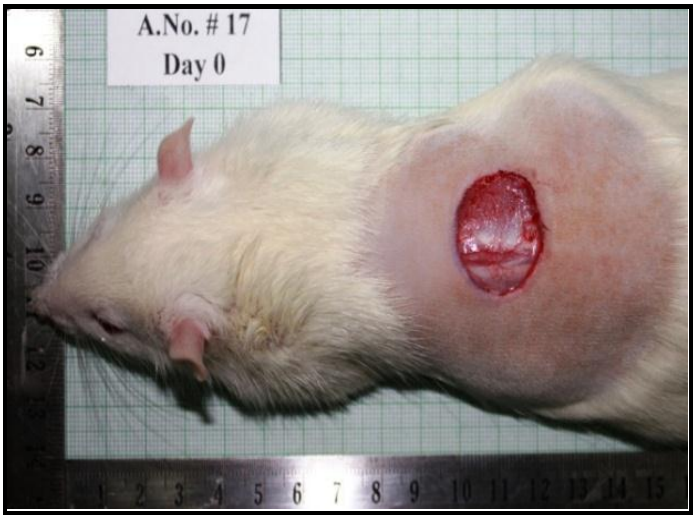


Figure 3

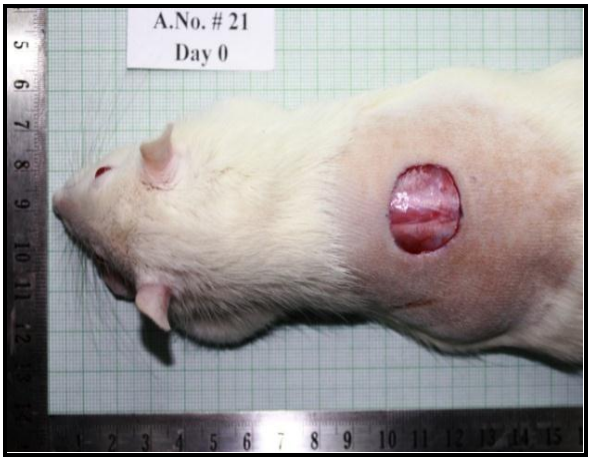


Figure 4

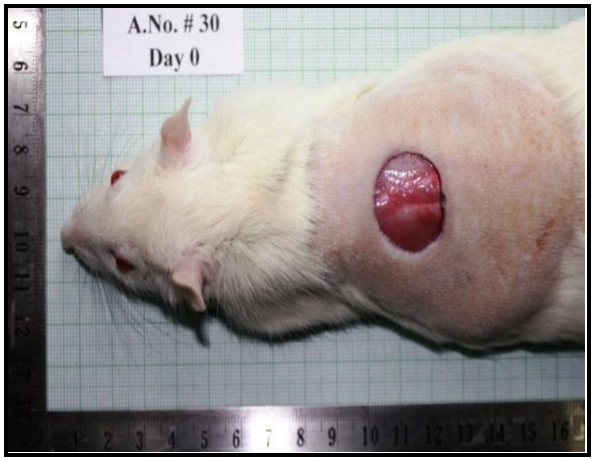


Figure 5

GROSS PHOTOGRAPHY OF WOUNDS ON DAY 15

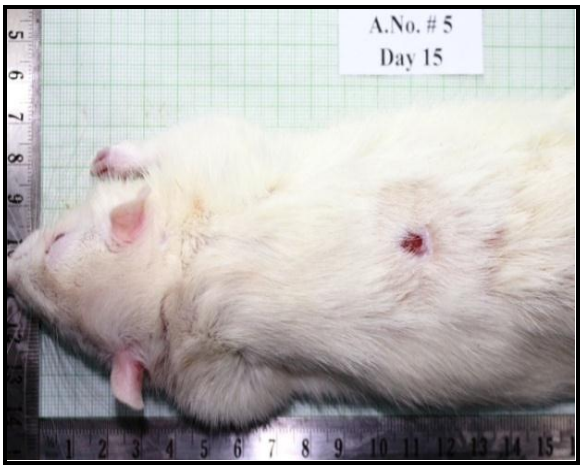
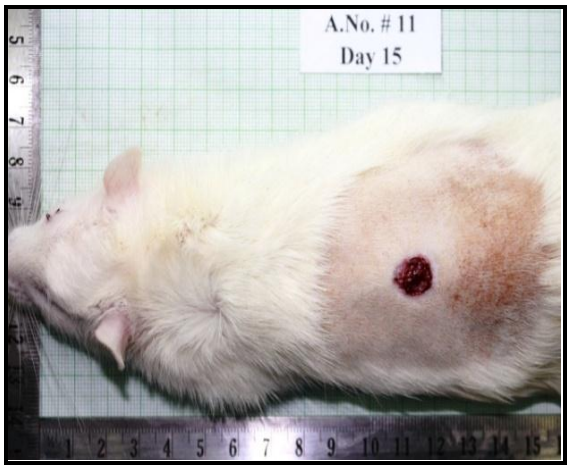


Figure 6



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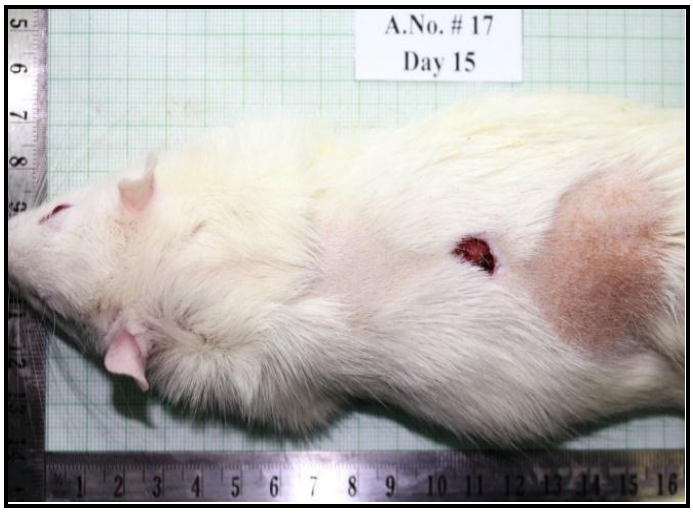

Figure 8

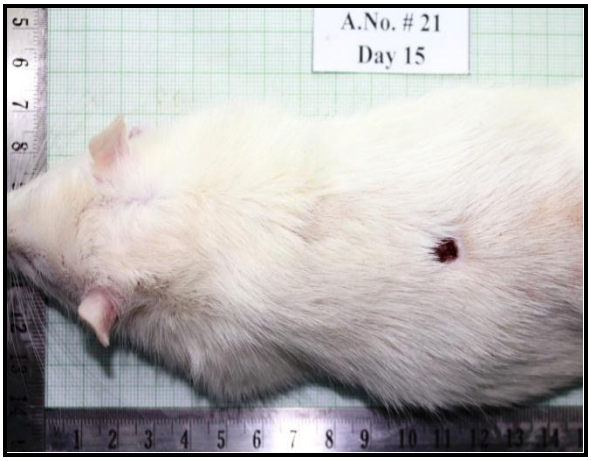

Figure 9

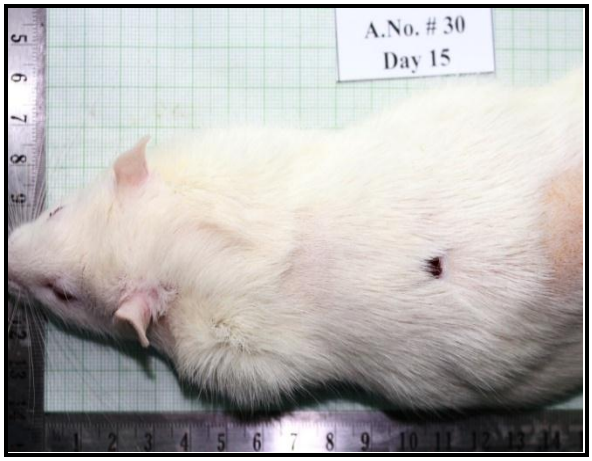

Figure 10

Figure: 1-10: Comparison of wounds from Group I - V on day 0 and 15.

4.5 Haematological Studies

The haematological parameters studied on day 15 revealed a slight increase in mean RBC (red blood cell) count in all the diabetic groups (group II, III, IV and V) as compared to the non diabetic control group I, however it was statistically significant only in group II and IV. Slight but statistically significant increase in the mean value of HGB (Haemoglobin) and HCT (Haematocrit) were also observed in group IV as compared to group I. There was a significant decrease in PLT (Platelet) count observed in diabetic control as well as in all the dose groups (group II, III, IV and V) as compared to the non diabetic control group I. There was a significant decrease in percent Eosinophils count observed in groups II and V and Basophils count in group V as compared to group I.

Although statistically significant, any of these parameters did not follow any pattern or dose response.

4.4 Coagulation Studies

The coagulation parameters *viz.* PT and APTT studied on day 15 did not reveal any significant changes within different treatment groups (group III, IV and V), when compared with the non diabetic control group I and diabetic control group II.

4.7 Clinical Chemistry

There was a significant increase in the mean Alanine Aminotransferase (ALT) in all the diabetic rats belonging to groups II, III, IV and V as compared to the non diabetic control

group I. However, mean ALT of group III, IV and V did not differ significantly from that of diabetic control group (group II). The values of these parameters are presented in Table 6.

Table 6: Summary of Clinical Chemistry.

Group	ALT	CREA	GLU	Na	K	Cl
I	45.33±5.32	0.45±0.08	101.3±13.19	136.6±1.17	3.267±0.22	100.1±1.63
II	101.5±29.34*	0.38±0.08	191.5±45.37	133.3±2.28*	3.400±0.52	94.15±1.81*
III	109.5±45.12*	0.33±0.05	200.0±86.27	135.0±2.11	3.333±0.78	96.18±2.40*
IV	118.5±49.21*	0.41±0.19	235.8±140.7	132.5±2.66*	3.133±0.58	93.78±0.58*
V	98.67±26.36*	0.40±0.06	177.7±86.61	133.6±2.19	3.583±0.54	94.53±0.54*

Values are Mean ± Standard Deviation,

* $p<0.05$

DISCUSSION

The present study was designed to investigate the potential of Amlodipine Besylate in diabetic wound healing. As per the results obtained in this study, Amlodipine Besylate improves the rate of wound healing but not in statistically significant manner.

All the diabetic rats belonging to diabetic control (group II) and diabetic treated (group III, IV and V) showed all the normal symptoms of DM like polyuria, polydipsia, polyphagia and weight loss. The mean body weight of rats belonging to group II, III and IV reduced significantly when measured on day 8 and 14. The reason for weight loss could be altered carbohydrate metabolism which promotes increased muscle wasting, structural degradation of proteins or loss of muscle protein.[13] An increase in the body weight of diabetic rats of group V suggested its protective role on muscle wasting which might be due to improvement in glycemic control and increased synthesis of structural proteins.[14] It is also mentioned that in non-adult diabetic rats, diabetic weight loss is not seen as their body system keeps on growing at this stage.[15]

The wound area and percent wound contraction measured on day 0, 2, 5, 9, 12 and 15 did not reveal any significant difference within different groups but the diabetic rats treated with Amlodipine Besylate at the dose 10 mg/Kg (group V) were better than all other groups indicating faster closure of wounds. It was also observed that on day 2, there was increase in wound size, which could be due to vasodilation.[16] Immediately after tissue injury blood vessels and lymphatics are disrupted and an initial phase of 5-10 minutes of vasoconstriction is followed by more persistent vasodilatation. Blood components are extravasated into wound cavity which can further lead to increased size of wounds.

The sporadic (although statistically significant) increase or decrease observed in various haematological parameters did not follow any pattern or dose response making their assessment inconclusive in the context of this study. In case of clinical parameters, there was significant increase in ALT in all the groups (group II, III, IV and V) as compared to non diabetic control (group I) rats. The increase in serum ALT activity was associated with liver toxicity, but it can also be elevated in association with skeletal muscle injury.[17, 18, 19] Further, it was also reported that expression of glucose transporter isoforms GLUT2 in tubular cells and hepatocytes interact with STZ and can cause damage to liver and kidney.[20] The level of electrolyte (Cl^-) decreased significantly as a result of osmotic diuresis with subsequent loss of water and electrolyte induced by glycosuria in diabetics.[21]

Based on the results under the circumstances of the study, it could be concluded that Amlodipine at the dose of 10 mg/Kg per oral seems to be a beneficial in the treatment of diabetic wound healing.

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