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# *IN-SILICO* AND *IN-VIVO* EVALUATION OF A TOPICAL FORMULATION CONTAINING CREATINE MONOHYDRATE FOR THE MANAGEMENT OF CHRONIC DIABETIC WOUNDS IN MALE WISTAR RATS

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**Keywords:**

Diabetic wound healing, Creatine monohydrate, *In-silico* study, *In-vivo* validation, Tissue regeneration, Collagen deposition, Histological analysis

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**ABSTRACT:** Chronic diabetic wounds are a common complication in individuals with diabetes and can lead to severe health issues if not treated quickly. Due to its capacity to improve cellular energy metabolism, Creatine monohydrate (CrM) has been suggested to possess potential health benefits. The effectiveness of a topical CrM formulation in healing chronic diabetic wounds in male Wistar rats was investigated in this research along with placebo, marketed formulation. Over the course of 21 days, the reduction of the wound and its fast pace of healing were observed. In contrast to the disease control group, the treatment group lesion size was significantly reduced and its wound closure rate was significantly higher. Increased collagen deposition and angiogenesis were also found by histological analysis in the CrM group, suggesting improved tissue regeneration. In conclusion, the topical CrM formulation showed encouraging benefits on wound healing and may have application in the therapeutic management of chronic diabetic wounds.

**INTRODUCTION:** Diabetes is a prevalent illness that exposes a considerable number of individuals to the threat of cardiovascular disease, blindness, chronic ulcers, kidney disease and other associated health issues [1]. According to the estimates by the International Diabetes Federation, the global incidence of new diabetes cases exceeds 96,000 annually [2].

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Diabetic chronic ulcers, typically in the lower limbs, present a major risk with potential for infections, amputations or even death and their healing is affected by factors like impaired inflammation, angiogenesis, glycation and neuropathy [3].

Cutaneous wound healing is complicated process encompassing four distinct phases: a) Hemostasis – to stop bleeding b) Inflammation – where inflammatory cells are recruited and promote healing c) Proliferation – for tissue repair) Remodeling to develop mature scar [4, 5, 6, 7]. Growth factors control angiogenesis and matrix re-organization [8, 9]. Keratinocytes, fibroblasts [10] and enzymes like matrix metalloproteinases (MMPs)

and tissue inhibitor of matrix metalloproteinases (TIMPs), play key roles [11]. Diabetes and wounds can cause oxidative stress, hinder healing and lead to complications [12]. Substances with antioxidant and anti-inflammatory properties may accelerate wound healing in diabetic individuals by reducing oxidative stress and inflammation [13].

The connection between nutrition and oxidative stress is well-established, with phytochemicals explored for diabetic wound healing [14]. Nutrition influences allostatic load and tissue repair, requiring adequate energy and protein for proper healing [15]. Current therapeutic options for diabetic wound healing are limited and exploring natural, safer molecules offers an appealing alternative [16].

Creatine, a natural compound, serves as a vital cellular energy source and can be obtained through diet [17]. It's known for enhancing muscle mass and has research interest for its potential in preventing neurodegenerative diseases due to its antioxidant properties [18]. Creatine monohydrate (CrM) is the commonly used form in dietary supplements and various food products [19]. Therefore, the goal of this research was to assess Creatine monohydrate's effects, particularly its antioxidant and anti-inflammatory properties, on wound healing in Streptozotocin induced hyperglycemic rats [20].

## MATERIALS AND METHOD:

**Materials:** Creatine monohydrate (98% purity) with molecular weight 131.14 was purchased from Yucca Enterprises, Mumbai, with appropriate certificate of analysis. Streptozotocin, nicotinamide of Merk and all other reagents were used of analytical grade.

### Methods:

***In-silico* Study:** Swiss ADME was used to predict the pharmacokinetic characteristics like absorption, distribution, metabolism and excretion. Established a compound-target network for better understanding of pharmacological process behind the impact of CrM. To create a drug effected target network diagram, information about compound and effective targets was input into Cytoscape 3.9.1 software. The identified targets were then subsequently transferred to a string database, and the species "Homosapiens" was chosen to build a protein-protein interactions.

**Design and Preparation of Formulation:** The formulation contains Creatine monohydrate (CrM), zinc oxide and arrowroot powder, the process involves several steps to ensure uniformity and quality. First, all the powders are carefully weighed and then they are passed through a 100# mesh size sieve. This ensures that the particles are of consistent size. Next, the ingredients were mixed in geometric proportion, creating a well-blended mixture. The mixture is then sterilized for hour to eliminate any potential contaminants. Once the sterilization process is complete, the powder is mixed thoroughly and transferred in a container. Multiple batches were prepared containing 2.5% (w/w), 5% (w/w) and 10% (w/w) of the drug mixture. Various evaluation parameters are assessed. This comprehensive evaluation ensures that the drug formulation meets the desired quality standards and is suitable for its intended use.

***In-vivo* Study:**

**Animals:** Adult male Wistar rats of around 8-10 weeks old (180-200 g) were ordered from Global Bioresearch Solutions Pvt. Ltd. Pune, India. The research was conducted in compliance with the guidelines set forth by CPCSEA guidelines and IAEC of Poona College of Pharmacy in Pune, India granted the approval for the experimental protocol (PCP/IAEC/2023/2-5).

**Acute Skin Irritation Analysis:** Male Wistar rats weight between 180-200 g were utilized for the study. After 24 to 72 hours the skin was evaluated for the sign of erythema, edema and inflammation ta assess any potential skin irritation caused by the formulation [21].

**Induction of Disease:** Diabetes was induced initially by injecting single i.p dose of 110 mg/kg body weight Nicotinamide in saline and after 15 minutes Streptozotocin was injected with dose of 65 mg/kg body weight emulsified in 0.1 M fresh cold citrate buffer (pH 4.5)[22]. After injecting rats were permitted to utilize 5% glucose solution to prevent hypoglycemic shock. After a period of 72 hours, fasting blood glucose level (BGL) was examined of the animals and BGL > 200 mg/dl were selected as diabetic [23, 24].

**Excision Wound Model:** When the wounds were created, animals were anesthetized with 35 mg/kg

intraperitoneal of thiopentone sodium. The circular wound of about 300 $mm^2$ was created with the help of surgical scissor. The rats were kept in separate cages for further experiments [25].

The study consist of five groups with 6 animals in every group and treatment was given topically.

**Normal Control (NC):** Healthy rat + Wound + Water.

**Diabetic Control (DC):** Diseased rat + Wound + Water.

**Marketed Formulation (NS):** Diabetic rat + Wound + Neosporin topical powder.

**F (5%):** Diabetic rat + Wound + Creatine monohydrate topical 5% w/w formulation.

**F (10%):** Diabetic rat + Wound + Creatine monohydrate topical 10% w/w formulation.

**Measurement of Wound area and Percent Contraction:** The changes in the wound area was examined by camera and tracing technique on following days starting from 0 to 4, 8, 12, 16 till 20th. Then percent wound contraction was evaluated with the formula [26]:

% Contraction of wound = (Initial area – Specific day area) / Initial area × 100

**Determination of Period of Epithelialization and Wound Index:** The endpoint for determining complete epithelialization was marked by the absence of any raw wound once the scab had naturally fallen off. The duration of epithelialization was measured as the number of days required for the entire healing process to be completed. The Wound index was assessed using a subjective scoring system, as described earlier [27].

**Histopathological Examination:** Tissue samples were collected from rat skin after the experiment to assess Histopathological changes. The samples were treated with 10% buffered formalin for fixation, processed and embedded in paraffin blocks. The sections were cut and stained with hematoxylin and eosin (H and E) [28].

**Hydroxyproline Content:** The segments of skin were weighed and the mixed along with phosphate buffer (0.1M, pH 7.4) and subject to homogenization on the ice for 15 minutes at 10,000 rpm. Supernatant of tissue homogenate was used for determination of hydroxyproline content as described [29].

**Statistical Analysis:** All the values were stated as Mean±SEM (n=6). Data analysis was conducted using GraphPad Prism 9.0 software. Each statistics was analyzed by one way or two-way ANOVA followed by Dunnett's or Bonferroni's test [30].

## RESULTS AND DISCUSSION:

***In-silico* Activity:** The network was created between the molecules, its gene targets and disease categories related to diabetes and wound healing. The summary statistics of network shows number of nodes is 20 and number of edges is 22. The network shows creatine monohydrate is linked with 6 genes and disease associated with these genes related to diabetic wound healing are 13 which is depicted in **Fig. 1.**

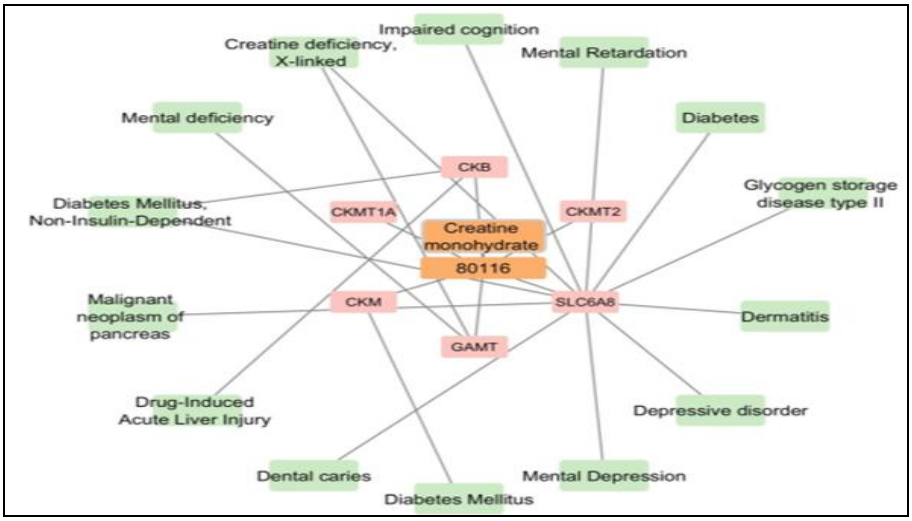


**FIG. 1: NETWORK PHARMACOLOGY**

**Evaluation of Formulation:** The physicochemical evaluation of the CrM formulation and the commercially available counterpart was conducted, and the results are presented in **Table 1**.

The pH values of formulations ranged from 5.5 to 6.3, which falls within the normal pH range for human skin. The CrM (10%) formulation closely resembled the properties of the marketed formulation. The angle of repose for the test formulation was found to be 33.69° and its porosity was 2.76%.

In comparison, the marketed formulation exhibited an angle of repose of 33.69° and a porosity of 2.52%. The Moisture content of the 10% formulation was determined to be 3.50%, while the marketed formulation had a moisture content of 2.70% respectively.

**TABLE 1: THE PHYSICOCHEMICAL EVALUATION OF DIFFERENT FORMULATIONS**

| Sr. no. | Parameters | C2 (5%) | C1 (10%) | NS (Neosporin) |
|---|---|---|---|---|
| 1 | Colour | White | White | White |
| 2 | Odour | Odourless | Odourless | Odourless |
| 3 | Appearance | Smooth | Smooth | Smooth |
| 4 | pH | 5.7 | 6.2 | 6.3 |
| 5 | Particle size (mm) | 0.149 | 0.149 | 0.149 |
| 6 | True density in g/cm$^3$ | 1.42 | 1.68 | 1.56 |
| 7 | Bulk density g/cm$^3$ | 0.66 | 0.58 | 0.62 |
| 8 | Tapped density g/cm$^3$ | 0.85 | 0.92 | 0.92 |
| 9 | Angle of repose | 33.69$^o$ | 33.69$^o$ | 33.69$^o$ |
| 10 | Porosity | 2.22 | 2.76 | 2.52 |
| 11 | Carr's Index | 22.35% | 36.95% | 32.60% |
| 12 | Hausner ratio | 1.28 | 1.58 | 1.48 |
| 13 | Moisture content | 3% | 3.50% | 2.70% |

**Acute Skin Irritation Study:** The study involved testing various doses of the test formulation to assess its acute toxicity and determine the appropriate therapeutic dose. Throughout the study, it was noted that concentrations up to 30% w/w of CrM formulation did not induce any alteration in behavior, itching of the skin, inflammation, swelling, erythema redness of the skin, irritation or instances of mortality. As a result, 5% and 10% concentration of the formulation were chosen for further investigation in subsequent study.

**Blood Glucose Level and Body Weight:** The **Fig. 2** depicts the blood glucose level of diabetic rats over time, showing a consistent increase as diabetes developed. However, there was no considerable change in blood glucose level observed among the various treated groups of diabetic rats ($p > 0.05$). Conversely, the blood glucose levels of non-diabetic rats differed significantly from those of the diabetic rats ($p < 0.05$). Additionally, it was found that topical application did not lead to a noteworthy decrease in glucose level in diseased rats ($p > 0.05$). There was a considerable reduction in body weight of diabetic wound control rats when compared with normal wound control rats as well as treated groups( $p < 0.05$).

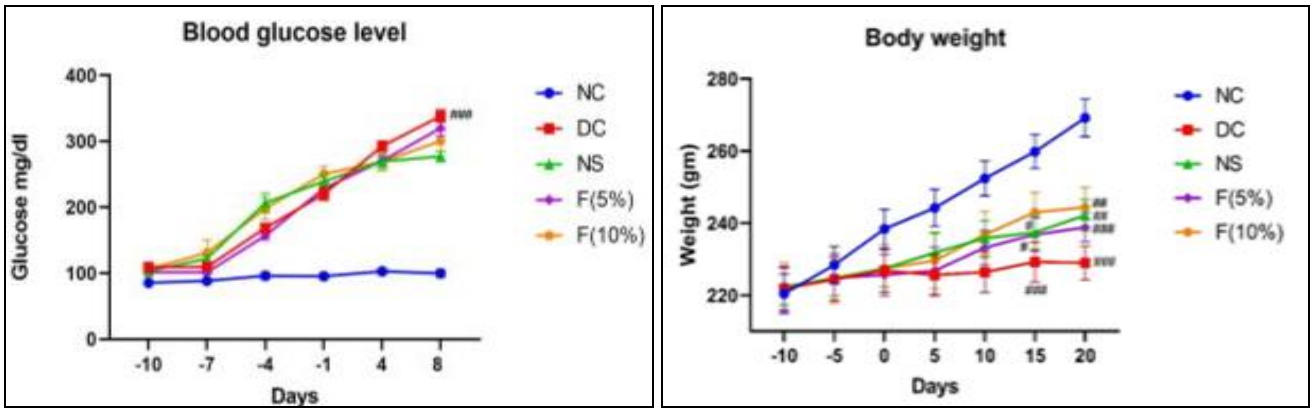


**FIG. 2: EFFECT OF CRM FORMULATION ON BLOOD GLUCOSE AND BODY WEIGHT**

**Wound area and %Wound Contraction:** The Wound healing progress was visually documented over 21 days, as shown in the **Fig. 3**. All experimental groups exhibited a remarkable reduction ($p>0.05$) in wound area ($mm^2$) and a considerable elevation ($p<0.05$) in %wound closure related to disease control group, on days 0, 4, 8, 12, 16, 20 as depicted in the **Fig. 4**. Quantitative measurement of the wound area confirmed that the %wound closure from day 12 in the disease control group was significantly different ($p<0.05$) from the normal control group. Conversely, all treated groups exhibited a significant difference ($p<0.05$) in %wound contraction correlated to the disease control group. By the 21st day post-wounding, the %wound closure in treated groups was 99.3±0.5%, respectively. Remarkably, the F (10%) treated group showed significantly higher wound healing compared to all other treated groups ($p < 0.05$). These results suggest that the inclusion of CrM in the formulation accelerates the wound recovery process by enhancing wound re-epithelialization.

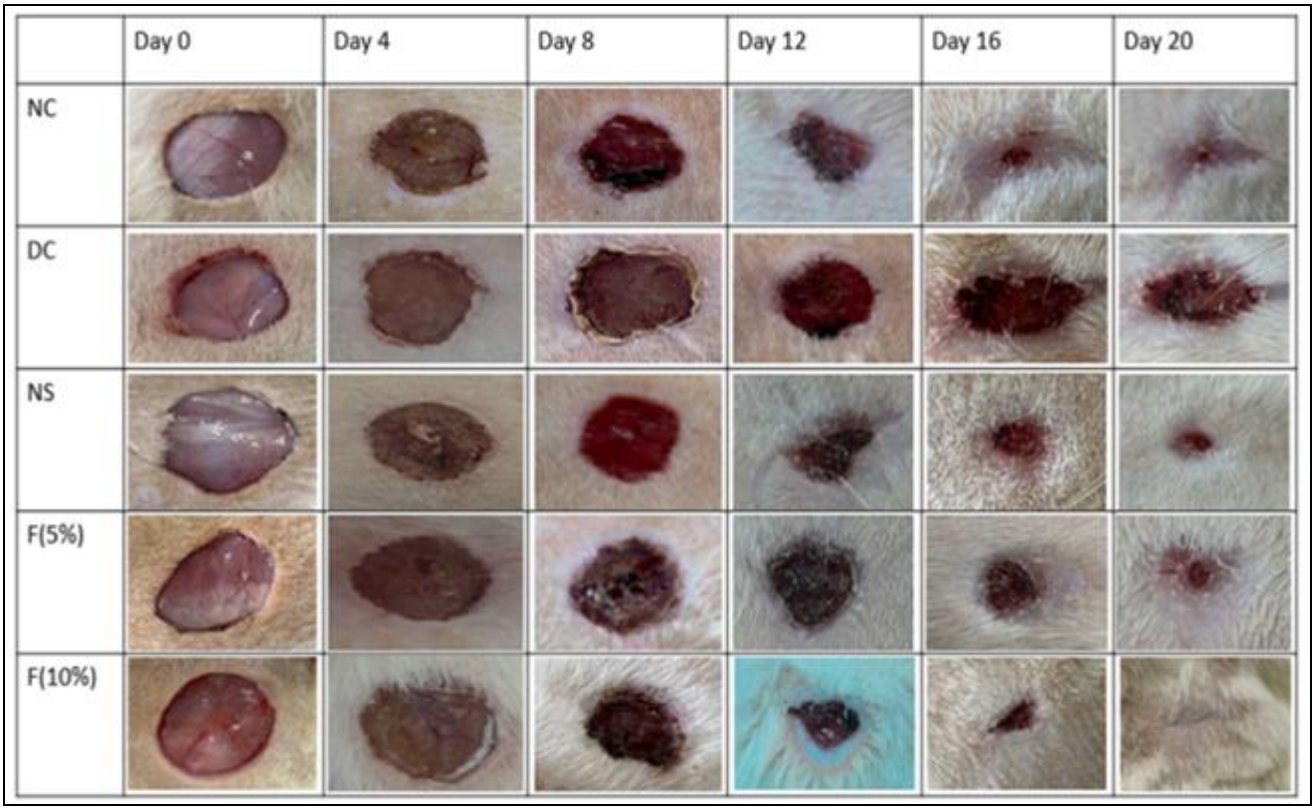


**FIG. 3: PHOTOGRAPHIC DEPICTION OF THE EFFECT OF CRM FORMULATION ON HEALING PHASE IN AN EXCISION MODEL**

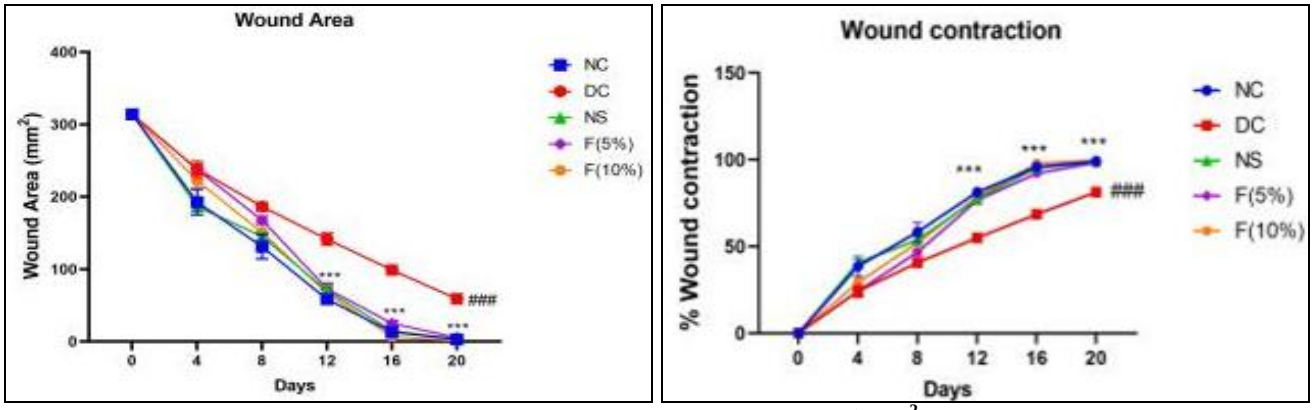


**FIG. 4: EFFECT OF CRM FORMULATION ON WOUND AREA ($MM^2$) AND % CONTRACTION**

**Epithelialization Period and Wound Index:** The **Fig. 5** presents the Mean±SEM (n=6) of the period required for epithelialization and wound index. In the group treated with the formulation, the

sloughing of the scab took about 20 days and resulted in no residual wound scar. However, in the disease group, the wounds remained partially unhealed. Throughout the experiment, all treated groups showed a superior wound index compared to that of disease control group, indicating better wound healing progress.

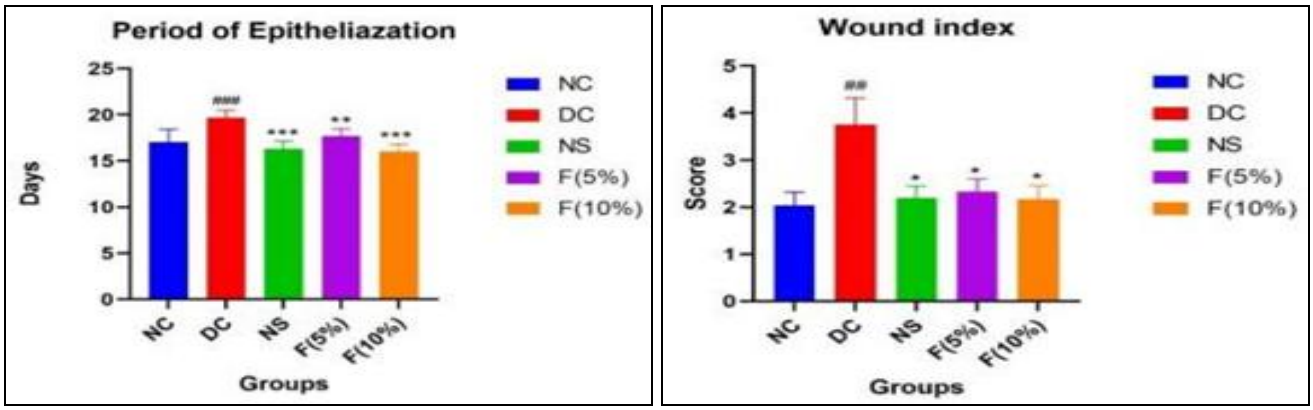


**FIG. 5: IMPACT OF CRM FORMULATION ON PERIOD OF EPITHELIALIZATION AND WOUND INDEX**

**Histopathology:** Histopathological assessment of wound tissue is of critical importance and outcomes presented in **Fig. 6** and **Table 2**. In the control group the production of fibrosis in the dermis, epithelization of tissues, and adnexa restoration was lagged as compared to Treated groups. Microscopic examination of skin tissue on the 21$^{st}$ day after injury in the normal control group revealed a high presence of cells showing inflammation. The control group rat skin had an early tissue epithelization and granulation tissue in addition with ulceration and edema and a high concentration of mononuclear inflammatory cells. The diabetic control group showed necrosis, less collagen fibers, pus cells and an average amount of inflammatory cells along with decreased re-epithelialization. The standard treated group/ neosporin revealed repaired structures, including well-formed, nearly regular epidermis, restored adnexa, and a dermis with significant fibrosis and collagen tissue. The formulation 5% and 10% treated exhibited significant fibrosis, a considerable quantity of granulation tissue, a low number of mononuclear inflammatory cells and the repair of adnexa.

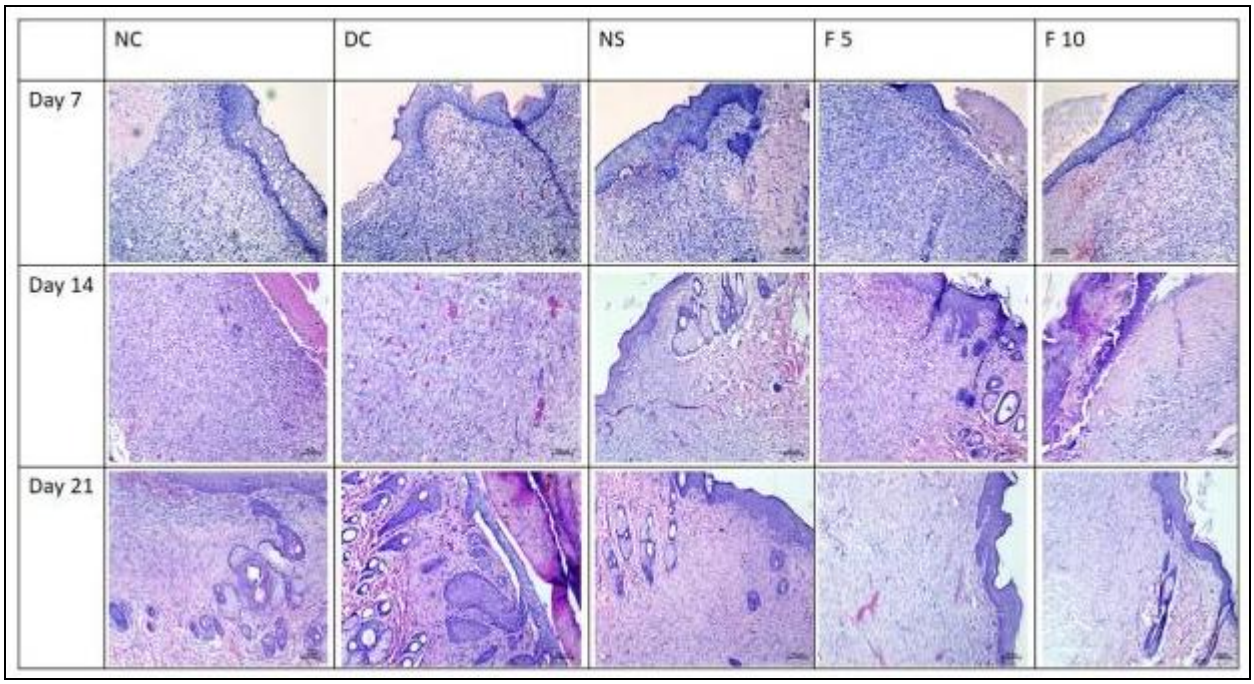


**FIG. 6: HISTOPATHOLOGICAL EXAMINATION OF SKIN OF MALE WISTAR RATS BY H AND E STAINING**

**TABLE 2: TISSUE HISTOPATHOLOGICAL DATA OF 21ST DAY OF POST WOUNDING**

| Group | Treatment | Re-epithelialization | Fibroblast deposition | Collagen deposition | Neovascula-rization | Inflammatory cells |
|---|---|---|---|---|---|---|
| I | NC | + | ++ | + | ++ | ++ |
| II | DC | - | - | - | - | +++ |
| III | Standard | ++ | ++ | ++ | ++ | + |
| IV | F (5%) | + | + | ++ | + | + |
| V | F (10%) | ++ | ++ | ++ | ++ | + |

Histological section represents (-) absent, (+) Mild, (++) Moderate, (+++) Extensive.

**Estimation of Hydroxyproline Content: Table 3** displays the hydroxyproline content in the wound tissues of the rat groups under examination. The group that received the 10% formulation exhibited the highest concentration of L-hydroxyproline. On the 21st day after injury, the hydroxyproline levels were notably elevated in the groups treated with the commercially available formulation and the 10% formulation when compared to the disease control ($p<0.05$).

**TABLE 3: EFFECT OF CRM FORMULATION ON ESTIMATION OF HRDROXYPROLINE CONTENT**

| Group | Hrdroxyproline (µg/ml) |
|---|---|
| NC | 38.70±1.78 |
| DC | 23.48±0.83 ### |
| NS | 36.82±0.76 *** |
| F 5% | 33.79±0.72 *** |
| F 10% | 36.68±1.41 *** |

**CONCLUSION:** Successful wound healing is characterized by the effective closure of wounds without any adverse effects. Our study focused on the wound healing phase in diabetic rats, and we observed promising results with the topical application of Creatine monohydrate (CrM). The use of CrM demonstrated strong wound healing effects, along with notable antimicrobial and antioxidant activities. The results of the wound closure assessments indicated that diabetic wounds treated with the CrM formulation showed superior wound closure capacity. Histological analysis further revealed increased collagen deposition and enhanced re-epithelialization in the treated wounds. Overall, our findings suggest that the topical application of CrM accelerates wound recovery through following key mechanisms. Stimulation of granulation tissue formation through collagen synthesis, facilitation of tissue remodelling via collagen replacement could be reasons for promotion of wound contraction. These positive effects indicate the potential of CrM as a beneficial treatment for enhancing chronic diabetic wound healing.

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**CONFLICT OF INTEREST:** Nil

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