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Research Article

Mesalazine based topical hydrogel formulation enhances anti-oxidant and cytokine activity in wounded STZ-induced mice

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Abstract

Background: The present study is targeted to elucidate the wound healing potential of mesalazine in STZ induced diabetic mice by comparing various antioxidant, pro-inflammatory cytokine levels and other wound healing parameters at 3,7 and 14th day.

Methods: Full thickness excisional wounds of 6mm size were created on the dorsal side of STZ induced mice and topical treatment of mesalazine based different hydrogels was applied for 14 days. Wound tissues were excised on day 3, 7 and 14 for various wound healing parameters.

Results: Delayed in wound healing was observed in diabetic group which eventually got accelerated after application of ethosome based mesalazine loaded hydrogel designated as D5 in study. Significant wound contraction rate was observed in D5 group and tissue hydroxyproline and tensile strength was also elevated after treatment with mesalazine loaded ethosomal hydrogel. The level of ROS was found to be significantly decreased in SOD, CAT and GSH experiment. LPO level were found to be elevated in D5 group. Finding of levels of pro-inflammatory cytokine suggested significant decrease and showed significant elevation in vascular endothelial growth factor (VEGF).

Conclusion: D5 group of ethosome based mesalazine loaded hydrogel showed promising results in controlling all the factors to their normal range and was effective in accelerating wound 51healing in diabetic mice.

Keywords: Diabetic wounds; hydrogels; antioxidants; cytokines; VEGF; Mesalazine

1. INTRODUCTION

Diabetes is a systemic metabolic disorder characterized by an impaired glucose metabolism. It can cause serious complications if uncontrolled [1]. One of the major complications arise from this disease is delayed diabetic wound healing. Wound healing is a magnificently synchronized and coordinated process of various metabolic components. It is usually a well-known fact that diabetic wounds do not heal well and timely. The optimum level and precise response of ROS, several growth factors and inflammatory cytokines are the key players during the process of wound healing. If any of these synchronization gets hurdled, it results in delaying of wound healing [2]. In case of diabetes, this process is interrupted due to various extrinsic and intrinsic factors including high blood glucose levels, reactive oxygen species, cytokine level disbalance, growth factors imbalance etc. [3]. In a normal individual, wound healing occurs in three major phases:1) inflammatory phase, where dead cells are being removed and stimulation of regeneration occurs, 2) proliferation phase, where migration of cells occurs to promote granulation, angiogenesis, re-epithelization and 3) remodeling and repair phase, where epidermis is perfectly regenerated. However, under diabetic condition, the inflammatory phase is prolonged causing increase in the levels of reactive oxygen species (ROS) and cytokines resulting in impairment in wound healing [4,5]. Therefore, easy and effective treatment strategy is needed to overcome this situation in diabetic patients to improve their quality of life.

Hydrogels have been known to be an excellent mode of topical treatment from many years. It provides moist wound environment thereby removing wound exudates [6]. It has 70-90% of water imbibing capacity making it resembles excellently like tissue environment. Various numbers of drug have been formulated and used to treat wounds due to their curative property [7-9]. Mesalazine on the other hand is US-FDA approved drug and the chosen drug candidate for this study due to its previous history of downregulating ROS levels and exerting anti-inflammatory effect to its surrounding [10]. Previous studies done with mesalazine on different disease showed to downregulate pro-inflammatory cytokines like IL-1β and TNF-α. The exact mechanism of mesalazine mode of action is by inhibiting cyclooxygenase enzyme which plays a key role in arachidonic acid metabolism [11, 12].

The present study demonstrates the abnormal status of ROS and pro-inflammatory cytokines and growth factors in diabetic mice which are one of the reasons in delayed diabetic wound healing. Through our study, we were able to show that the use of previously prepared mesalazine based topical treatment could benefit the cause. The overall study focuses on two aspects: first aspect was development of mesalazine

loaded Carbopol based hydrogel for topical treatment. The second aspect focuses on wound healing efficacy of prepared hydrogel by performing various in-vivo tests. The preparation of mesalazine loaded hydrogel and its characterization has been done in previous part of the study. Therefore, we aimed to extend our novel findings of mesalazine based ethosomal hydrogel as topical mode of drug delivery to treat diabetic wounds by comparing the levels of various wound healing factors namely ROS, pro-inflammatory cytokines and pro-angiogenic growth factor between diabetic and non-diabetic conditions. The study also aimed to determine the levels of hydroxyproline and tensile strength of diabetic and non-diabetic wound tissue comparing pre and post treatment by respective hydrogels.

2. MATERIALS AND METHODS

2.1. Animals

Swiss albino mice aged 4-6 weeks (20-25 g) were procured from Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (Haryana) India. Mice were housed in a controlled temperature 25 ±1℃ and of 55 ±5% humid environment with 12-hour light-dark cycles and were given food and water accurately. The experimental procedures were performed under the protocol approved by the Institutional Animal Ethics Committee BV/IAEC/Dec.2018/5.

2.2. Induction of Diabetes

Diabetes will be induced using streptozotocin injections intraperitonially (40mg/kg) for 5 consecutive days. Confirmation of diabetes was done 48 hours after the last dose of streptozotocin (STZ) and will be further confirmed for development of diabetes via GOD-POD kit [13].

2.3. Excisional wound creation and experimental protocol

The superficial wound of ethically permitted size: 6 mm diameter was created on the back of induced diabetic mice [14]. Immediately after acclimatization mice were divided into experimental groups of non-diabetic (Nd) and diabetic (D). The grouping was categorized into (1) Uninjured (2) Injured control (3) Injured and treated with plain Carbopol (4) Injured and treated with simple mesalazine hydrogel (5) Injured and treated with ethosomal hydrogel incorporated with mesalazine of non-diabetic and diabetic respectively. After the creation of wound, the topical treatment of hydrogel was given to respective groups for 14 days daily once a day at fixed time. The wound tissue was excised at 3, 7 and 14th day respectively after sacrificing the mice to further study the efficacy of mesalazine in all the treatment groups.

2.4. Evaluation of blood glucose level

100µl of blood was withdrawn from tail of mice and GOD-POD kit (Span diagnostics Ltd., India) was used to evaluate glucose level. Glucose level were examined and compared at days 3, 7 and 14 after the topical administration of respective hydrogels.

2.5. Visual photographs and Wound contraction study

Wound area from diabetic and non-diabetic mice, after the treatment with different hydrogels was photographed everyday with standard camera from standard height from day 1 to 14. Multiple images of animals in control and treatment group respectively were obtained and wound contraction area was estimated by Image Pro v. 6 software for determining epithelialization time and percentage of wound contraction was compared to size of wound on the initial day.

2.6. Estimation of hydroxyproline

Evaluation of hydroxyproline content was done on 3, 7 and 14th day of wound excision. The wound tissue was excised and stored at -20℃ till analysis is done. Tissue samples were oven dried at 50-60℃ and was hydrolyzed in 6M HCl (100µl for 1mg dried tissue). The hydrolyzed tissue was stored at 130℃ for 3 h in sealed tubes. The hydrolyzed lysate was then neutralized with 2.5 M NaOH. 2ml of sample was taken and chloramine-T was incorporated for oxidation for 20 mins. The reaction was put to halt by addition of 1ml of 3.15 M of per chloric acid. The resultant sample was allowed to stand undisturbed for 5 min. Ehrlich reagent measuring 1ml was incorporated and the mixture is shaken and incubated in water bath at 60℃ for 20 min. development of pink colour takes place which is further measured at 550nm by UV-visible spectrophotometer. The amount of hydroxyproline present in the tissue is evaluated from the standard curve from pure L-hydroxyproline [15].

2.7. Assessment of tensile strength

Tensile strength of excised tissue was evaluated by instrument called tensiometer. In brief, one end of the tissue was tied to instruments end with the help of fish line wire. The other end was tied to fish line wire passing through pulley to which quantifiable weights are attached [16]. The weights evaluated the total tension applied to break the tissue by which tensile strength was calculated by given formula:

Tensile strength (gm/cm^2) =
breaking load (gm) / cross sectional area of tissue (cm^2)

2.8. Determination of levels of anti-oxidant enzymes

2.8.1. Determination of Superoxide Dismutase (SOD) Activity

To determine the activity of SOD, 25 µl of tissue supernatant was taken and incorporated into reaction mixture which consists of phosphate buffer 5mM, methionine 100mM, EDTA 1mM, riboflavin 10mM and nitro blue tetrazolium (NBT) 450mM. incubation of 30 min was done in light and blue colour appearance was observed which was measured spectrophotometrically at 560nm [17].

2.8.2. Determination of Lipid Peroxidation (LPO) activity

LPO assessment was done by adding 2µl of tissue supernatant to 1.5ml of 20% acetic acid, 0.2 ml of 8.1% SDS and 1.5 ml of 0.85% thiobarbituric acid. Further, 4ml of distilled water was incorporated into the reaction mixture and heated to 95℃ in water bath for 60 min. post heating, the reaction mixture was cooled down and proceeded for centrifugation at 10,000 rpm for 10 mins and measured spectrophotometrically at 532 nm [18].

2.8.3. Determination of catalase (CAT) activity

Tissue was homogenized and supernatant was taken and incorporated into 1ml reaction mixture which contains 0.8mM 3% H_2O_2 and 0.1 mM sodium phosphate buffer maintained at pH 7. The activity was evaluated by decomposition of H_2O_2 / min/mg protein. Further, the absorbance was recorded at 240nm spectrophotometrically [19].

2.8.4. Determination of Glutathione Reductase (GSH)

Glutathione reductase activity was assessed by the method described by Mokrasch and Teschke [20]. Briefly, formic acid was incorporated into tissue supernatant and the process was further proceeded by centrifuging the mixture to 10,000 rpm for 10 mins. The deprotenized tissue supernatant was added to tube containing buffered formaldehyde [1:4 (v/v) 37% formalin: 0.1 M Na_2HPO_4]. Each tube was previously filled with sodium phosphate buffer (0.1M, 5Mm EDTA). o-

phthalaldehyde was incorporated further in to mixture the fluorescence was measured after incubation of 45 min at room temperature at excitation and emission wavelength at 345 and 425nm respectively.

2.9. Determination of level of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and growth factor (VEGF)

Levels of cytokines in different wound tissues (post hydrogel application) of mice was evaluated using enzyme-linked immunosorbent assay (ELISA). Mice wounds was harvested on day 3, 7 and 14 with surgical procedure and homogenized in cold phosphate buffer saline (10 µL/mg wound tissue) supplemented with protease inhibitor cocktail by using a standard homogenizer and then sonicated and centrifuged at 10,000 rpm for 20 minutes at 4°C. Supernatants was utilized for the ELISA of tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β) and interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) by mouse specific ELISA kits (R&D Systems, USA).

2.10. Histopathological examination

The wound tissue was excised on 3, 7 and 14th day respectively and were fixed in 10% formaldehyde solution for 1 hr and stored in 70% ethanol. The fixed samples were embedded in paraffin wax and processed for 5µm thick sections. The thick waxed sections were further stained with hematoxylin and eosin (H&E) and were observed under bright field microscope (Olympus compound microscope, Japan).

2.11. Statistical Analysis

The results were performed in triplicate and expressed as Mean± SD. Control groups were compared with treated groups and results were analyzed by using two-way ANOVA. SNK (Student Newman- Keuls) test was performed for analyzing the results by software Sigma stat 3.5. the data with $P \leq 0.001$ and $P \leq 0.01$ were considered significant.

3. RESULTS

3.1. Evaluation of blood glucose level

After the application of mesalazine loaded hydrogels, there was a significant decrease ($P \leq 0.001$) observed in both D3 and D4 group i.e., treated with simple mesalazine loaded hydrogel and treated with ethosome based mesalazine loaded hydrogel respectively shown in Fig. 1. However, no significant change was observed in carbopol treated group.

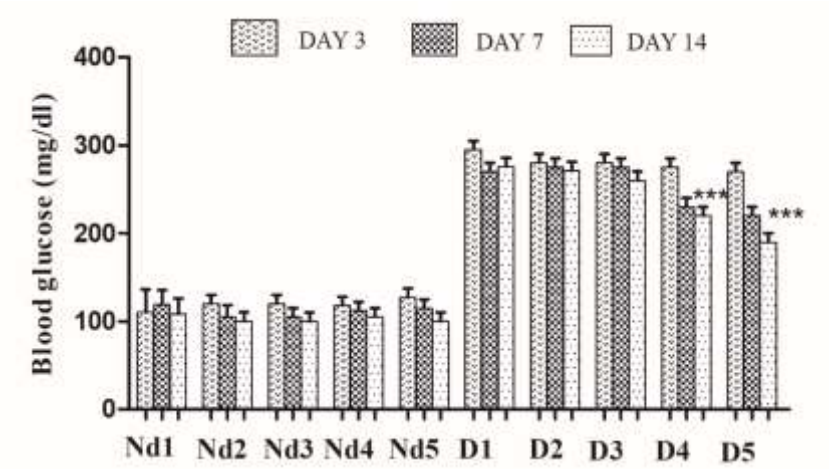

Figure 1: Comparitive evaluation of blood glucose level after application of mesalazine based different hydrogels of treatment groups. Each bar signifies mean±SD of experiments in triplicate. ***$P \leq 0.001$, **$P \leq 0.01$ and *$P \leq 0.01$ implies significant values using SNK test followed by two-way ANOVA.

3.2. Visual photographs and Wound contraction study

Photographs were taken from day 0 to day 14 from standard camera at fixed height throughout the study. The result showed significantly greater wound contraction in treatment group D5 i.e., treatment with ethosome based mesalazine loaded hydrogel at 14th day compared to D4 group i.e., treated with simple mesalazine hydrogel and control. D4 showed optimal wound contraction at day 14 in comparison with D5 group. The external observational assessment of wound contraction in all the experimental group is shown in Fig. 2.

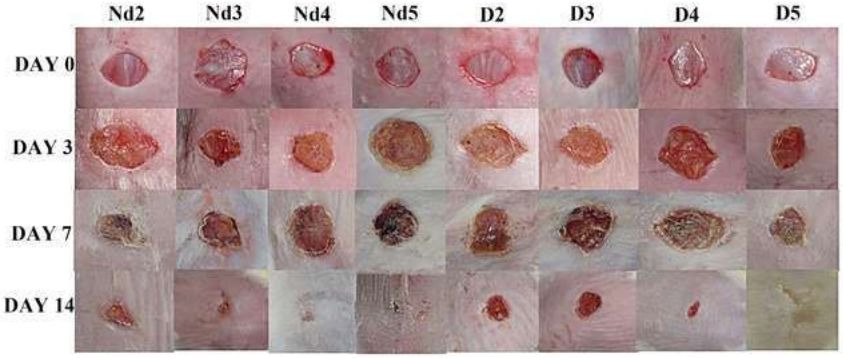

Figure 2: Visual photographs at 3, 7 and 14th days of treatment representing the effect of Mesalazine based ethosomal hydrogel (D5) in STZ-induced mice.

3.3. Estimation of hydroxyproline

The effect of ethosomal hydrogel (D5 group) on hydroxyproline level (r^2=0.96) increased in a dose dependent manner. The total hydroxyproline content of D4 and D5 group was found to be significantly higher (P≤0.001 and P≤0.01) on 14th day when compared to non-treated control group and treated with Carbopol. The result of hydroxyproline content of all the experimental groups is shown in Fig. 3.

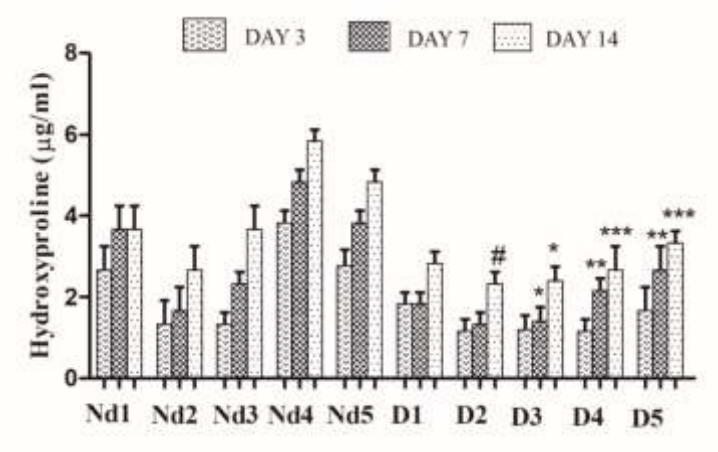

Figure 3: Effect of mesalazine loaded different hydrogels on hydroxyproline level of non-diabetic and diabetic wound tissue on day 3, 7 and 14, expressed in (µg/ml). Bar represents mean ± SD values of triplicate results. ***P≤0.001, **P≤0.01 and *P≤0.01.

3.4. Assessment of tensile strength

The result of tensile strength of all the groups on 14th day is shown in Fig. 4. the tensile strength of animals treated with ethosome base mesalazine hydrogel (D5) and simple mesalazine loaded hydrogel was significantly higher (P≤0.001 and P≤0.01) than that of control group and treated with Carbopol.

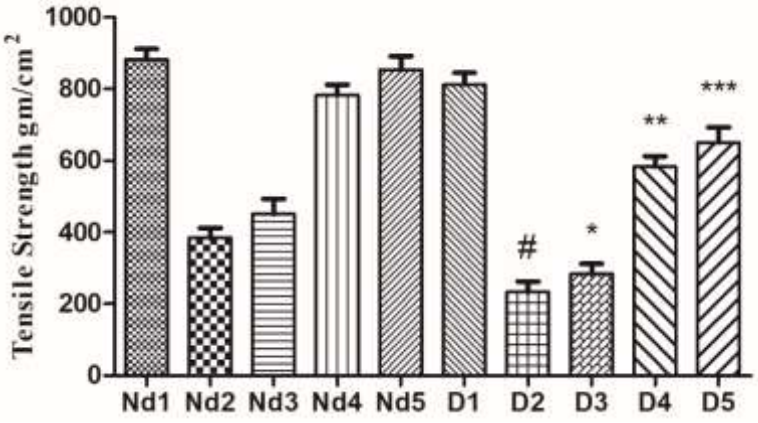

Figure 4: Tensile strength (gm/cm^2) of different treatment groups at 14th day of excised tissue. ***P≤0.001, **P≤0.01 and *P≤0.01, n=3.

3.5. Determination of levels of anti-oxidant enzymes

3.5.1. Determination of Superoxide Dismutase (SOD) Activity

Increase in the level of ROS is the major factor in delayed diabetic wound healing. Level of SOD in different treatment groups of diabetic mice was found to be significantly decreased (P≤0.001) when compared to non-diabetic group and is shown in Fig. 5(A). However, the SOD activity was found to be significantly increase on day 7 and 14 in diabetic group treated with ethosomal hydrogel (D5) when compared to control (D2) and placebo group (D3). D4 also showed increase in SOD level making it optimally significant (P≤0.01) in comparison with D2 and D3 group.

3.5.2. Determination of Lipid Peroxidation (LPO) activity

LPO level in terms of MDA (nM/mg protein) in all the treatment and non-treatment groups is shown in Fig. 5(B). The level of LPO in D5 and D4 group showed significant decrease (P≤0.001) on 14th day from wounding when compared with control (D2). There was significant increase in lipid peroxidation level of diabetic groups in comparison with non-diabetic group. However, there was no significant change observed in D3 group treated with plain Carbopol.

3.5.3. Determination of catalase (CAT) activity

The level of CAT was seen to be recovered significantly from control group (D2) to topical treatment by ethosomal hydrogel (D5) group and the result is expressed in Fig. 5(C). Diabetic mice treated with ethosome based mesalazine loaded hydrogel shows significant increase (P≤0.001) in level of CAT at 7 and 14th day post treatment. D4 group also has shown to be recovered optimally (P≤0.01) and no significant change was observed in D3 group treated with plain Carbopol.

3.5.4. Determination of Glutathione Reductase (GSH)

The wound tissue from treated and non-treated groups showed significantly increase in GSH level in diabetic group treated with both hydrogels. Group D5 and D4 showed significant increase ($P \leq 0.001$and $P \leq 0.01$) in the level of GSH when compared to injured control. However, diabetic mice treated with plain Carbopol (D3) showed no significant change when compared to control. The result of GSH level of all the treatment groups in both diabetic and non-diabetic mice is represented in Fig. 5(D).

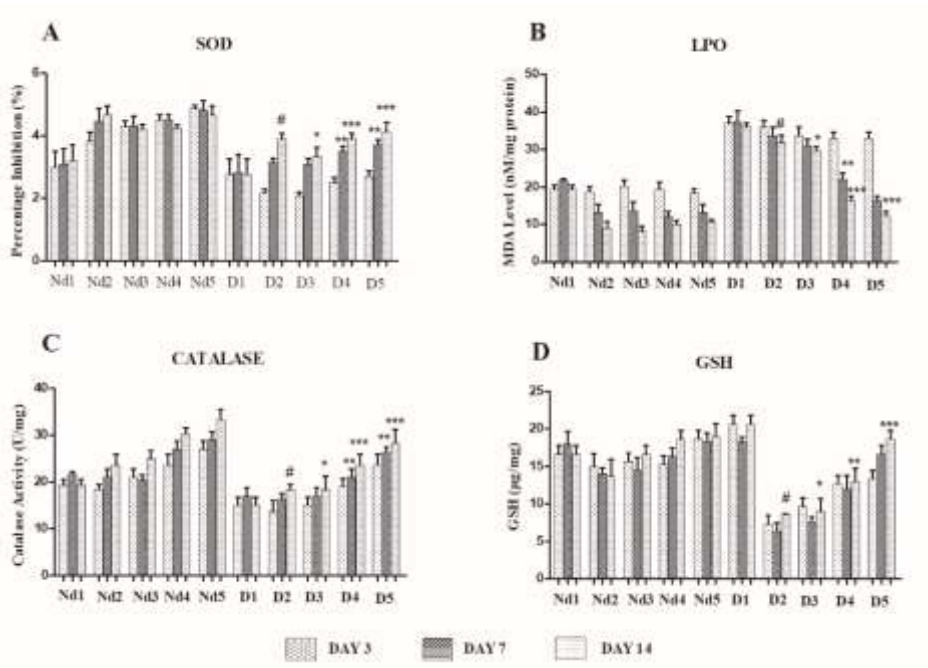

Figure 5: Effect of different treatment groups on antioxidant levels of non-diabetic and diabetic wound tissue. A) SOD (% inhibition) B) LPO (measured by malonaldehyde expressed in nM/mg protein) C) CATALASE (U/mg) and D) GSH (µg/mg). Each bar represent mean ±SD where ***$P \leq 0.001$, **$P \leq 0.01$ and *$P \leq 0.01$, n=3.

3.6. Determination of level of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and growth factor (VEGF)

Levels of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and pro-healing cytokine VEGF was evaluated by ELISA and the results are shown in Fig. 6. The levels of IL-1β, IL-6 and TNF-α were significantly decreased ($P \leq 0.001$) in D4 and D5 treatment group when compared with control represented in Fig. 6(A, B and C) respectively. D3 treatment group showed no significant change when compared with control. In contrast the level of VEGF in D4 and D5 group showed a significant increase [Fig. 6(D)]. This finding is consistent with the histopathological study.

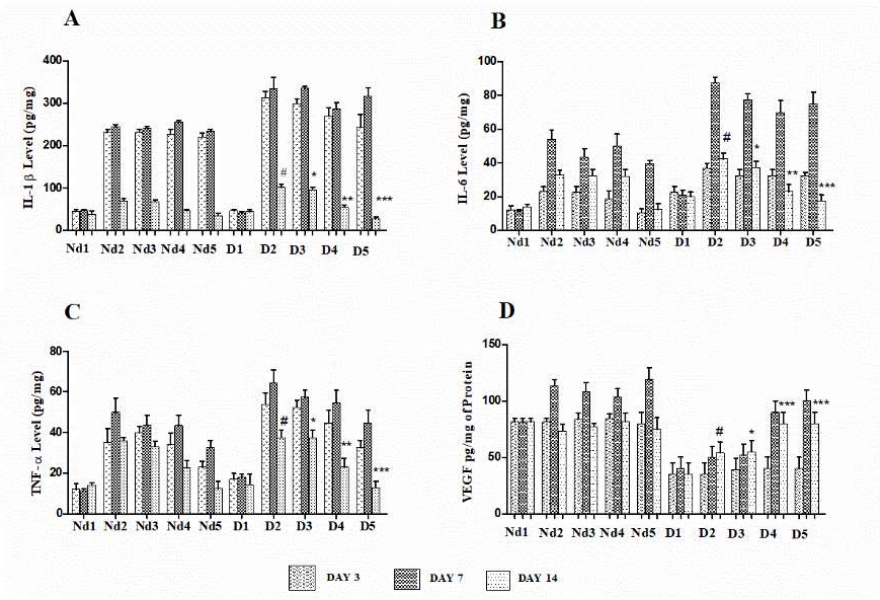

Figure 6: Comparison in levels of pro-inflammatory cytokine and growth factor at day 3, 7 and 14 in non-diabetic and diabetic mice. Above figure represents levels of A) IL-1β B) IL-6 C) TNF-α and D) VEGF. Each bar represents mean ± SD values of triplicate results. ***$P \leq 0.001$, **$P \leq 0.01$ and *$P \leq 0.01$.

3.7. Histopathological examination

The efficacy of treatment group on wound tissue was analyzed microscopically in wound tissue sections stained with H&E at 3, 7 and 14th day. Histopathological findings of treated and non-treated group showed significant recovery as shown in Fig. 7. Group D5 and D4 has shown regeneration of epidermal layer. H&E analysis of control and Carbopol group (D2 and D3) showed minimal detectable changes in epidermal integrity.

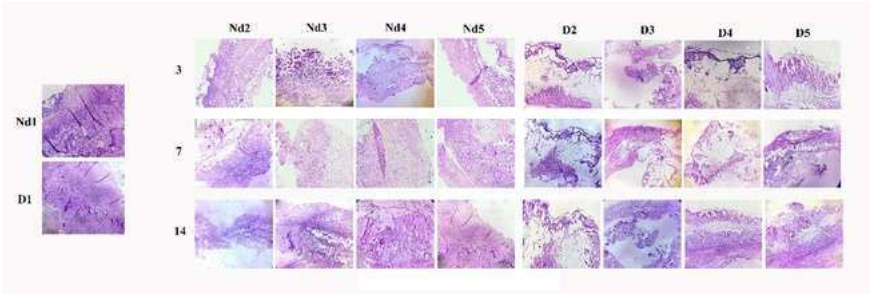

Figure 7: Histopathological comparison on day 3, 7 and 14th showing levels of epidermal regeneration in all the treated and non-treated of non-diabetic and diabetic mice.

4. DISCUSSION

The present study demonstrates the abnormal status of ROS and pro-inflammatory cytokines and growth factors in diabetic mice which are one of the reasons in delayed diabetic wound healing [21]. Through our study, we were able to show that the use of previously prepared mesalazine based topical treatment could benefit the cause. The overall study focuses on two aspects: first aspect was development of mesalazine loaded Carbopol based hydrogel for topical treatment. The second aspect focuses on wound healing efficacy of prepared hydrogel by performing various in-vivo tests. The preparation of mesalazine loaded hydrogel and its characterization has been done in previous part of the study.

Diabetic wound model was developed by injecting streptozotocin to adult swiss albino mice weighing 20-25gm and creating ethically permitted 6mm wound on the dorsal shaved region. After creation of wound, study was further proceeded to evaluate various factors including wound contraction assessment, tensile strength and evaluation of levels of various reactive oxygen species and pro-inflammatory and pro-healing cytokines. Evaluation of level of hydroxyproline and histopathological assessment was also done to determine the efficacy of mesalazine in topical form to treat diabetic wounds.

Two types of hydrogels were used to study the efficacy of mesalazine on diabetic wounds. The first hydrogel is simple Carbopol based mesalazine incorporated hydrogel (D4) and the second one is ethosome based mesalazine incorporated hydrogel (D5). During the assessment of wound contraction, D5 showed significantly greater wound contraction and D4 showed optimum wound contraction. Whereas, D3 mice treated with plain Carbopol shows no significant change in the study. Uninjured group was taken in whole study to compare the levels of each metabolic components in relaxed uninjured status. Level of hydroxyproline is marked as tissue collagen indicator [13]. After treatment of 14 days, collagen deposition was found abundantly on D5 group with increased level of hydroxyproline. The same result is showed by D5 group in tensile strength. Interruption in the levels of ROS leads to impairment of diabetic wounds. Inflammation is the major step involved in the healing of diabetic wound. The inflammatory phase is recognized by removal of dead cells and enables regeneration to occur. Prolonged inflammatory phase under diabetic cycle can form a malicious cycle, delaying wound healing [4, 5]. In the study done to determine the levels of various ROS enzyme activities, we observed decrease in the level of SOD, CAT, GSH and increase in the level of LPO in diabetic wounds which were eventually restored after topical administration of ethosome based mesalazine loaded hydrogel (D5). Wound healing is also highly regulated by growth factors and cytokines [22]. The pro-inflammatory cytokine level of IL-1β, IL-6, TNF-α was found to be significantly decreased by D5 group and optimally decreased by D4 group. Increase in the level of VEGF indicates increase angiogenesis. This stimulates formation of new blood vessels, accelerating wound healing phase [23]. The findings of VEGF study showed decrease in the level of VEGF under diabetic condition but got restored when treated with mesalazine based hydrogel. Group D5 significantly increases the VEGF level of diabetic mice whereas D4 also increases the level in optimum basis. Histology comparative evaluation of 3, 7 and 14th day showed re-epithelization in treated group of both diabetic and non-diabetic states.

Mesalazine in previous studies has been proven to restore the antioxidant levels in colonic mucosa [24]. The results showed by mesalazine in two different hydrogel form indicates its effectiveness in topical treatment. Mesalazine in the simple hydrogel form D4 group showed slightly/optimally decreased results when compared to ethosome based hydrogel. This is due to the fact that ethosomes is an excellent transdermal drug delivery carrier. The high ethanolic and phospholipid content makes the lipid bilayer soft and enhances the drug permeation rate through deeper layers of skin.

5. CONCLUSION

Mesalazine was developed in two different forms and both showed significant results in controlling the metabolic components under diabetic conditions. However, D5 group showed excellent results when compared to D4 group due to the fact that ethosome based topical treatment provide enhanced drug delivery to deeper layer. Also, the study was performed to compare the levels of various metabolic components including ROS, pro-inflammatory cytokines and growth factors, hydroxyproline level and tensile strength at 3, 7 and 14 days post treatment under non-diabetic and diabetic conditions. D5 mesalazine group showed promising results in controlling all the factors to their normal range and was effective in accelerating wound healing in diabetic mice.

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Conflict of interest: The authors declare no conflict of interest.

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