

## Research Article

# Sodium Butyrate Plus EGF and PDGF-BB Aids Cutaneous Wound Healing in Diabetic Mice

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Received 10 August 2015; Revised 13 October 2015; Accepted 13 October 2015

Academic Editor: Paul J. Higgins

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Topical application of growth factors is known to aid defective and/or delayed wound healing in diabetic patients. In this study, the effect of topical application of sodium butyrate (Na-Bu), EGF, and PDGF-BB was analysed on the acute cutaneous wound healing in streptozotocin (STZ) induced diabetic mouse model. Two cutaneous wounds were created, one on each of the dorsolateral sides of the diabetic mice. Na-Bu, EGF, and PDGF-BB were applied to the wound either individually or in various combinations. The wound healing was monitored visually and scored as percentage wound closure. The tissue samples were collected from the wound site at 1, 7, and 14 days after wounding from the treated and untreated diabetic wounds and analysed for the levels of EGF-R,  $\beta$ -PDGF-R, HDAC1, p21, and phosphorylated and hypophosphorylated pRb proteins. Our results indicate that application of EGF plus PDGF-BB at the initial stages followed by subsequent addition of Na-Bu along with these growth factors helps wound healing in diabetic mice. It appears that, in addition to cell proliferative agents, a cell differentiation agent, Na-Bu, is necessary for diabetic wound healing. Topical application of EGF plus PDGF-BB along with Na-Bu could be developed as therapeutic agents to treat and manage human diabetic wounds.

## 1. Introduction

Diabetes is a common chronic metabolic disorder and is characterised by hyperglycaemia and loss of glucose homeostasis. Patients with diabetes suffer from nonhealing chronic wound, especially foot ulcers, which remains as a global burden even after rigorous treatments with available means [1, 2]. Topical application of growth factors, epidermal growth factor (EGF), and platelet-derived growth factor-BB (PDGF-BB) accelerated wound healing process in normal cases [3–5] and their positive effects are also shown in diabetic wound healing to certain extent [6–10].

Streptozotocin (STZ) produces mild to severe diabetes in a dose-dependent manner when it is given as intravenous (i.v.) or intraperitoneal (i.p.) injections in animal model. Therefore, STZ induced diabetic animals are used to study diabetes related biological properties including wound healing [11].

Sodium butyrate (Na-Bu) is a nontoxic, naturally occurring fatty acid and it relieves epithelial inflammation [12].

Na-Bu induces cell differentiation [13] and is also an inhibitor of histone deacetylase (HDAC) [14, 15]. In this study, the effect of Na-Bu along with the growth factors EGF and PDGF-BB on the cutaneous wound healing process was assessed in STZ induced diabetic mice model. The results indicate that Na-Bu when provided along with growth factors can aid cutaneous wound healing in diabetic mice. Our data also shows that Na-Bu might be necessary to induce cell differentiation during diabetic wound healing process. It appears that, in addition to cell proliferative agents such as growth factors, cell differentiation agent like Na-Bu is mandatory for efficient healing of diabetic wound. Use of growth factors plus Na-Bu in a time-bound manner could be developed as a therapeutic agent to manage/treat diabetic wound in human.

## 2. Materials and Methods

*2.1. Experimental Mice Model.* This study was approved by the National Institute of Mental Health and Neurosciences

TABLE 1: Details of growth factors, Na-Bu, and the various antibodies used in this study.

Product	Supplier	Product number	Molecular weight of the band in western blots	Final concentration/dilution (for antibody) used
EGF	Sigma-Aldrich, St. Louis, MO, USA	E-6135	—	10 ng
PDGF-BB	Sigma-Aldrich, St. Louis, MO, USA	P-3201	—	15 ng
Sodium butyrate (n-butyric acid, sodium salt)	Sigma-Aldrich, St. Louis, MO, USA	B-5887	—	2 mM
Anti-EGF-R antibody Ab-6	Oncogene Research Product, Boston, MA, USA	Catalogue number PC98	185 kDa	1:1000
Anti- $\beta$ -PDGF-R antibody	BD Biosciences (BD Pharmingen), Singapore	Catalogue number 554288 (BD Biosciences) 15746E (BD Pharmingen)	180 kDa major; 160 kDa minor	1:1000
Anti-HDAC1 antibody	Sigma-Aldrich, St. Louis, MO, USA	H3284	65 kDa	1:2000
Anti-P21 <sup>WAF1/Cip1</sup> antibody	Sigma-Aldrich, St. Louis, MO, USA	P1484	21 kDa	1:1000
Anti-pRb antibody	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	Rb (Rb1): sc-73598	110 to 114 kDa	1:500
$\gamma$ -tubulin antibody	Sigma-Aldrich, St. Louis, MO, USA	T5326	50 kDa	1:1000
Alkaline phosphatase conjugated protein A (secondary antibody)	Calbiochem, Nottingham, UK	539251	—	1:1000

(NIMHANS) Animal Ethics Committee, certificate number AEC/50/314/HG. Four-week-old male Swiss albino mice were obtained from Central Animal Research Facility (CARF), NIMHANS, Bangalore, India. They were kept in polypropylene cages in a well-ventilated experimental room at 25–30°C, relative humidity of 40–60%, and 12-hour light/dark cycle for an additional 2 weeks for acclimatization. Before wounding, 5 mice were placed in each cage and after wounding single mouse was placed in each cage. The mice were fed standard pelleted food from Godrej India Ltd., Mumbai, India, and water *ad libitum*. The diet consisted of approximately 70% carbohydrate, 20% protein, 7% fat, and 3% salts and other essential vitamins and minerals [3–5].

**2.2. Streptozotocin-Induced Diabetes.** Streptozotocin (STZ) was obtained from Sigma-Aldrich, St. Louis, USA. Fresh STZ was prepared every time and used within 5–10 minutes. Briefly, 10 mg/mL STZ stock was prepared fresh in cold 0.01 M citrate buffer pH 4.5. Just before injection, the stock solution was diluted to a concentration of 1 mg STZ in 200  $\mu$ L of the same citrate buffer. Six-week-old mice were starved for 20 hours prior to the first injection of STZ. Appropriate volume of STZ depending on the body weight of each mouse was injected intraperitoneally each day for 5 consecutive days. In general, 1 mg STZ in 200  $\mu$ L of citrate buffer was injected in a mouse weighing approximately 25 g which equals 40 mg STZ/kg body weight [11]. The control group was injected with 200  $\mu$ L of citrate buffer alone. The blood glucose level was measured weekly from the last day of STZ injection.

The blood samples were collected from the tail vein and deproteinized, and the glucose level was estimated spectrophotometrically by glucose oxidase/peroxidase method [16]. For quick estimation of glucose level, a glucometer kit, Accu-Chek, from Hoffman-La Roche (India) was used.

**2.3. Wounding.** The wounds were created ten days after the last injection of STZ. Approximately 1 cm long full thickness wounds were created on both of the dorsolateral sides of each mouse. Briefly, the mice were anesthetised individually with Halothane-soaked cotton (Piramal Healthcare Limited, India), in a glass jar with loose fitted, holed lid. The surface of the mice was cleaned with ethanol, hairs were removed, and two wounds measuring 1 cm long, one on each of the dorsolateral sides, were created with a sharp surgical blade [3–5].

**2.4. Growth Factors and Na-Bu Treatments.** The details of growth factors used in this experiment are given in Table 1. Recombinant human PDGF-BB and recombinant mouse EGF were obtained from Sigma-Aldrich (St. Louis, MO, USA) as lyophilised powder and they were reconstituted in a solution containing 4 mM HCL and 0.1% Bovine Serum Albumin (BSA) according to the protocol given by the supplier. Aliquots of appropriate volumes were stored at –20°C and they were thawed and used only once [3–5]. A stock solution of 200 mM Na-Bu was prepared in 1 mL water as described previously [13, 17]. Optimum concentrations of growth factors EGF (10 ng) and PDGF-BB (15 ng) and Na-Bu

TABLE 2: Time-line for STZ treatment, wounding, topical application of growth factor(s) and Na-Bu, and tissue harvest.

Day 0	D1 to D5	D6 to D15	D16 W1	D17 W2	D18 W3	D19 W4	D20 W5	D21 W6	D22 W7	D23 W8	D24 W9	D25 W10	D26 W11	D27 W12	D28 W13	D29 W14
20 h STR	STZ	—	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT
G0	G1	G7 G15	G16						G22							G29
			TH1						TH7							TH14

Day 0 is the beginning of the experiments. The animals were 6 weeks old and they were starved (STR) for 20 h prior to the induction of diabetes. STZ, daily injection of streptozotocin at a concentration of 40 mg/Kg body weight for consecutive 5 days (D1 to D5). Ten days (D6 to D15) after the last STZ injection, the wounds (W) were created and the day of the wounding is taken as W1; TT, various topical treatments as given in Table 4. G, measurement of glucose levels on the days after beginning the experiment; TH, days when the tissues were harvested for western blotting.

(2 mM) [3–5] were applied directly using a micropipette to the wound as described previously [3–5]. A schematic representation of the time course of STZ injection, wounding, growth factor and Na-Bu application, glucose estimation, and tissue harvest is given in Table 2. The growth factors of appropriate concentrations in 10  $\mu$ L volume were applied immediately after wounding to each set of 6 mice as follows: (i) a set of nondiabetic mice were kept as control; (ii) a set of STZ injected, untreated mice were kept as diabetic control; the wounds of these two groups were treated daily with 10  $\mu$ L of the diluent used to reconstitute growth factors; the remaining sets of experimental mice were treated daily as follows: (iii) 2 mM Na-Bu for 14 days; (iv) 10 ng of EGF for 14 days; (v) 10 ng EGF for the first 2 days followed by 10 ng EGF + 2 mM Na-Bu for the next 12 days; (vi) 15 ng PDGF-BB for 14 days; (vii) 15 ng PDGF-BB for the first 2 days followed by 15 ng PDGF-BB + 2 mM Na-Bu for the next 12 days; (viii) 10 ng EGF + 15 ng PDGF-BB for 14 days; (ix) 10 ng EGF + 15 ng PDGF-BB for the first 2 days followed by these growth factors plus 2 mM Na-Bu for the next 12 days.

**2.5. Monitoring.** The wounds were monitored daily and scored visually for wound closure and new growth of irregular mass of granular tissues. The wound closure was measured and expressed as percentage.

**2.6. Tissue Sample Collection and Lysis.** The experimental and control groups consisted of 6 mice each. The day of wounding was considered as day 1. The tissues from the wound sites were collected on days 1, 7, and 14 after wounding. The day 1 samples were collected after wounding followed by respective treatments for approximately 30 minutes to 1 hour (Table 4). The pooled tissue samples from 2 mice were suspended in 100  $\mu$ L of lysis buffer consisting of 10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1.2% Triton X-100, 2 mM phenyl methyl sulfonyl fluoride (PMSF), 0.15 U/mL aprotinin, and 10  $\mu$ g/mL leupeptin (Sigma-Aldrich, St. Louis, MO, USA). The tissues were homogenised on ice using a Polytron (Kinematica, Switzerland) 30 seconds  $\times$  5 with 1 minute cooling in between strokes. The lysates were clarified by centrifugation at 8,000 g for 10 minutes at 4°C. The protein contents were quantified spectrophotometrically from the clear supernatants and analysed by western blotting.

**2.7. Western Blotting.** One hundred fifty micrograms of tissue lysates in 10  $\mu$ L volume was mixed with 10  $\mu$ L of protein sample buffer (sc-24945, Santa Cruz, Biotechnology, CA, USA), subjected to separation on a 7.5% SDS-polyacrylamide gel (PAGE) and transferred to PVDF membrane (Sigma-Aldrich, St. Louis, USA) using a Bio-Rad (Australia) semidry blotter. The nonspecific binding sites on the membrane were blocked in a buffer with BSA, incubated for 2 hrs in primary antibody, and washed and incubated with AP-conjugated secondary antibody (1:1000) for 1 hour at room temperature on a rocker platform. Details of the antibodies and dilutions are given in Table 1. Then, the membranes were washed and the bands were visualised with 1-Step NBT/BCIP (Pierce, Rockford, IL, USA) according to the protocol provided by the supplier. The band intensities were calculated from the membranes with Bio-Rad Gel-Doc system with quantity one software. The Broad Range Marker was used for molecular weight determination (sc-2361, Santa Cruz Biotechnology, California, USA). Duplicate gels were run and one of them was incubated with  $\gamma$ -tubulin primary antibody and used as internal loading control [13, 17].

**2.8. Data Analysis.** The software Statistical Package for Social Sciences (SPSS) version 16 was used for data analysis. The data for glucose level in blood are obtained from three sets of experiments and expressed as mean  $\pm$  standard error of mean (SEM). The levels of EGF-R, PDGF-R, and HDAC1 proteins in the untreated, nondiabetic control mice were normalised to the  $\gamma$ -tubulin loading control and it was taken as 1. Using this value as a standard, the levels of proteins in untreated diabetic control were calculated. The levels of these proteins in the treated wounds were compared to the untreated diabetic control wound and expressed as fold increase. The highest value for p21 was found in untreated diabetic control and after normalisation with  $\gamma$ -tubulin loading control it was taken as 1. The level of p21 in the treated wounds was compared to the untreated diabetic control wound and expressed as fold decrease. The pRb protein levels (110 plus 114 kDa bands) were first normalised to the  $\gamma$ -tubulin; the total pRb level was taken as 100%. The level of phospho-pRb (114 kDa band) was calculated in the nondiabetic control and untreated and treated diabetic wounds as compared to the total pRb and expressed as percentage [13, 17]. The levels of

TABLE 3: Level of glucose at various time points.

Sl. number	Day of the experiment	Level of glucose in control mice ( $n = 5$ ); mg/dL	Level of glucose in STZ injected mice ( $n = 5$ ); mg/dL
1	G0	131 $\pm$ 2.0	132 $\pm$ 2.1
2	G1	133 $\pm$ 1.5	131 $\pm$ 1.8
3	G7	132 $\pm$ 1.8	215 $\pm$ 1.5
4	G15	132 $\pm$ 2.0	298 $\pm$ 1.9
5	G16	133 $\pm$ 2.1	323 $\pm$ 2.5
6	G22	131 $\pm$ 1.7	334 $\pm$ 2.0
7	G29	132 $\pm$ 2.1	325 $\pm$ 2.3

G followed by number in days are as mentioned in Table 2. The data were calculated from 3 sets of experiments; the standard error of mean was determined and expressed as  $\pm$ ; there was no change in the blood glucose level in the growth factors and/or Na-Bu treated diabetic mice as compared to the untreated diabetic mice.

various proteins were obtained from three sets of experiments and the SEM were calculated. These data were analysed using one-way analysis of variance (ANOVA) and Student's *t*-test. The *p* value of  $<0.05$  is considered statistically significant.

### 3. Results

The power analysis showed that data obtained from use of 6 mice in each group was statistically significant.

**3.1. Level of Glucose in Control and Treated Mice.** The physiologically normal level of 130 to 133 mg/dL of glucose was present in the nondiabetic control mice. Two days after the last STZ injection, the glucose level was 215 mg/dL which represented the hyper glycaemic condition. Two weeks after the first injection of STZ, the glucose level was more than 300 mg/dL which confirmed diabetic condition of these mice and it was maintained for the following 2 to 3 weeks (Tables 2 and 3). The experiments were conducted during this period when the mice were in diabetic condition. There was no change in the blood glucose level between the growth factors and/or Na-Bu treated diabetic mice as compared to the untreated diabetic control mice (Tables 2 and 3).

**3.2. Effect of EGF, PDGF-BB, and Na-Bu on Wound Closure.** The percentage of wound closure was measured 14 days after wounding. Individual application of either EGF or PDGF-BB partially healed the diabetic wound with 40% and 55% of wound closure, respectively. The combination of EGF + Na-Bu and PDGF-BB + Na-Bu caused only 35% and 60% wound closure, respectively. The combination of EGF + PDGF-BB lead to 55% wound healing and had no additive effect. The combination of EGF + PDGF-BB for the first two days followed by these growth factors plus Na-Bu for the next 12 days produced the best wound healing and more than 95% wound closure. Topical application of Na-Bu alone had deleterious effect on diabetic wound healing process with less than 5% wound closure. The nondiabetic control wound

healed completely by day 14 after wounding. The untreated diabetic control wounds closed less than 10% and remained unhealed for up to a month after wounding (Table 4).

**3.3. Effect of Growth Factors and Na-Bu on EGF-R Protein Level.** The untreated diabetic wound had trace level of EGF-R protein as compared to the nondiabetic control wound. Topical application of EGF or PDGF-BB or their combination resulted in approximately 4- to 6-fold increase in the level of EGF-R protein in the diabetic wound tissues as compared to the untreated ones and it was statistically significant with  $p < 0.05$ . The peak level of EGF-R protein was found on day 7 after wounding. Combination of EGF plus Na-Bu resulted in 2.5-fold decrease in the level of EGF-R on days 7 and 14 as compared to that of EGF application alone. Application of PDGF-BB alone or in combination with EGF resulted in a 2-fold higher level of EGF-R protein on day 1 as compared to that of EGF application. PDGF-BB plus EGF application did not show any additive increase in EGF-R level. PDGF-BB plus Na-Bu or EGF plus PDGF-BB plus Na-Bu did not affect the EGF-R level significantly as compared to the PDGF-BB application. Na-Bu application alone had negligible effect on the EGF-R protein level (Figures 1 and 2).

**3.4. Effect of Growth Factors and Na-Bu on  $\beta$ -PDGF-R Protein Level.** The untreated diabetic control wound had trace level of  $\beta$ -PDGF-R protein as compared to the nondiabetic control. Topical application of EGF and PDGF-BB individually or in combination resulted in approximately 4- to 6-fold increase in the level of  $\beta$ -PDGF-R protein as compared to the untreated diabetic control wound and it was statistically significant with  $p < 0.05$ . The peak level of  $\beta$ -PDGF-R protein was found on day 7 after wounding in these cases. Combination of EGF plus Na-Bu reduced the level of  $\beta$ -PDGF-R on days 7 and 14 by 2.5-fold as compared to that of EGF application alone and it was statistically significant. PDGF-BB plus EGF application did not show any additive increase in  $\beta$ -PDGF-R level. PDGF-BB plus Na-Bu reduced the  $\beta$ -PDGF-R level by 2-fold on day 14 as compared to that found on day 7. Application of EGF plus PDGF-BB or EGF plus PDGF-BB plus Na-Bu produced the peak level of  $\beta$ -PDGF-R on day 7 and it did not change significantly on day 14. Application of Na-Bu alone caused little change in the level of  $\beta$ -PDGF-R protein level (Figures 1 and 3).

**3.5. Effect of Growth Factors and Na-Bu on the Expression of HDAC1 Protein.** The nondiabetic control had maximum level of HDAC1 protein on days 1 and 7 and it decreased by half on day 14. The untreated diabetic wound had a 6-fold decrease in the level of HDAC1 protein on days 1, 7, and 14 after wounding as compared to the nondiabetic control. EGF application produced a 6-fold increase in HDAC1 protein level on day 1 and it gradually decreased to 3-fold on day 14 (Figures 1 and 4). The combination of EGF plus Na-Bu lead to a 5-fold decrease in HDAC1 protein level on day 7 as compared to the EGF application and it remained unchanged on day 14. PDGF-BB application alone produced a 6-fold increase in HDAC1 protein level on days 1 and 7 and it

TABLE 4: Wound closure in control and treated groups.

Serial number	Growth factor, Na-Bu application	Percentage wound closure on day 14 after wounding
(i)	Nondiabetic untreated control	100%
(ii)	Diabetic untreated control	<10%
(iii)	Diabetic wound + 2 mM Na-Bu daily from day 1 to day 14	<5%
(iv)	Diabetic wound + 10 ng EGF; daily, day 1 to day 14	40%
(v)	Diabetic wound + 10 ng EGF (for first 2 days); 10 ng EGF + 2 mM Na-Bu (for the following 12 days)	35%
(vi)	Diabetic wound + 15 ng PDGF-BB daily from day 1 to day 14	55%
(vii)	Diabetic wound + 15 ng PDGF-BB (for 2 days); 15 ng PDGF-BB + 2 mM Na-Bu (for the following 12 days)	60%
(viii)	Diabetic wound + 10 ng EGF + 15 ng PDGF-BB daily from day 1 to day 14	55%
(ix)	Diabetic wound + 10 ng EGF + 15 ng PDGF-BB (first 2 days) followed by the same growth factors + 2 mM Na-Bu daily from day 2 to day 14	>95%

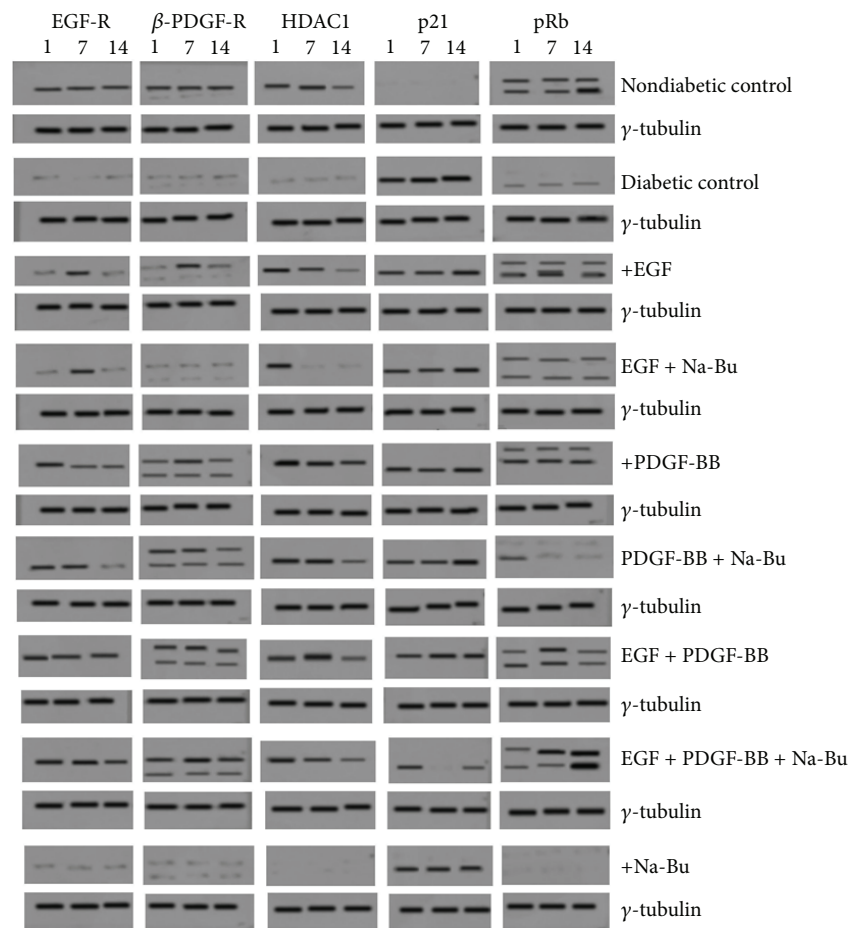


FIGURE 1: Western blot analysis of various proteins in the control and treated wound. The proteins are as marked on the top. The controls and various treatments are given on the right. The number is the days of tissues harvested for protein analysis. 1, day of wounding, 7, seven days after wounding, and 14, fourteen days after wounding. The  $\gamma$ -tubulin control is shown separately below each panel. Details of the antibodies and dilutions are given in Table 1.

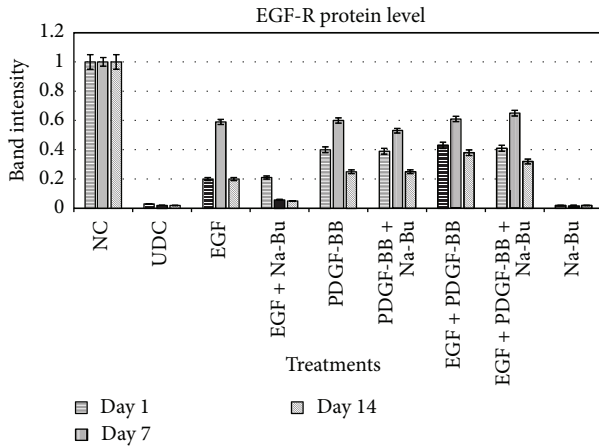


FIGURE 2: Effect of various treatments on the level of EGF-R protein. The various treatments are given in the  $x$ -axis. The band intensity is given in  $y$ -axis with Standard Mean Error. The horizontal line is day 1, vertical line is day 7, and diagonal line is day 14 after the wounding when the tissue was collected. NC, normal control; UDC, untreated diabetic control; the growth factor and Na-Bu treatments are as given below each panel.

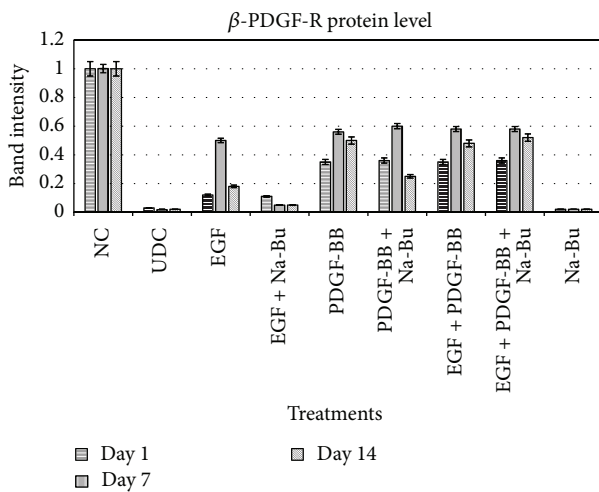


FIGURE 3: Effect of various treatments on the level of  $\beta$ -PDGF-R protein. The various treatments are given in the  $x$ -axis. The band intensity is given in  $y$ -axis with Standard Mean Error. The horizontal line is day 1, vertical line is day 7, and diagonal line is day 14 after the wounding when the tissue was collected. NC, normal control; UDC, untreated diabetic control; the growth factor and Na-Bu treatments are as given below each panel.

was statistically significant with a  $p < 0.05$  and on day 14 it decreased slightly. Application of PDGF-BB plus EGF produced approximately 6-fold increase in HDAC1 protein level on days 1 and 7 but it decreased to 3-fold on day 14. The combination PDGF-BB plus Na-Bu or EGF plus PDGF-BB plus Na-Bu caused a peak level of 6-fold increase in HDAC1 protein on day 1 and it gradually decreased to approximately 2- to 3-fold on day 14. The Na-Bu treated diabetic wound had no effect on the level of HDAC1 protein (Figures 1 and 4).

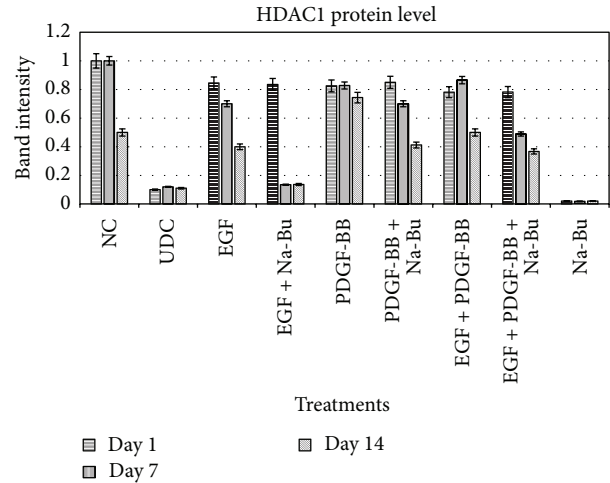


FIGURE 4: Effect of various treatments on the level of HDAC1 protein. The various treatments are given in the  $x$ -axis. The band intensity is given in  $y$ -axis with Standard Mean Error. The horizontal line is day 1, vertical line is day 7, and diagonal line is day 14 after the wounding when the tissue was collected. NC, normal control; UDC, untreated diabetic control; the growth factor and Na-Bu treatments are as given below each panel.

**3.6. Effect of Growth Factors and Na-Bu on p21 Protein Expression.** The diabetic wound tissue had approximately 6-fold increase in the level of p21 protein as compared to the non-diabetic control wound. Topical application of EGF or PDGF-BB and their combination with Na-Bu reduced the p21 protein level to approximately 2.5-fold on days 1 and 7 and 1.5-fold on day 14 as compared to the untreated diabetic wound and it was statistically significant with  $p < 0.05$ . The combination of EGF plus PDGF-BB reduced the p21 protein level to half of that found in untreated diabetic control wound and this level was maintained on days 1, 7, and 14 after wounding. The combination of EGF plus PDGF-BB plus Na-Bu caused a drastic 5- and 4-fold decrease in the level of p21 protein on days 7 and 14, respectively, as compared to the untreated diabetic control wound and it was statistically significant with  $p < 0.05$ . The p21 protein level in the Na-Bu treated wounds was almost similar to that of untreated diabetic wound (Figures 1 and 5).

**3.7. Effect of Growth Factors and Na-Bu on the Level and Phosphorylation of pRb.** The nondiabetic control wound had approximately 4-fold higher level of total pRb and 50% of it was found in phosphorylated form on days 1 and 7 as compared to the untreated diabetic control. On day 14, only 20% of total pRb was found as phosphorylated form and the remaining 80% was hypophosphorylated. The untreated diabetic control had about 5% of total pRb in phosphorylated form. EGF or PDGF-BB application resulted in a 3- to 5-fold increase in the level of total pRb protein and approximately 20% of it was found in phosphorylated form in the diabetic wound as compared to the untreated diabetic control (Figures 1 and 6). Application of EGF plus Na-Bu or PDGF-BB plus Na-Bu did not result in any significant change in the level of total pRb protein in the diabetic wound as compared to

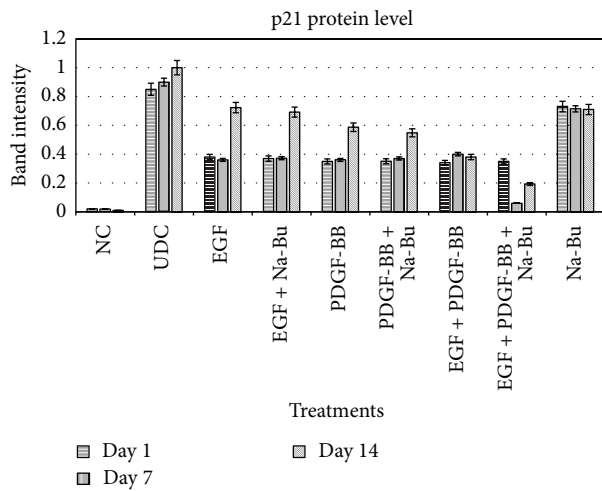


FIGURE 5: Effect of various treatments on the level of p21 protein. The various treatments are given in the x-axis. The band intensity is given in y-axis with Standard Mean Error. The horizontal line is day 1, vertical line is day 7, and diagonal line is day 14 after the wounding when the tissue was collected. NC, normal control; UDC, untreated diabetic control; the growth factor and Na-Bu treatments are as given below each panel.

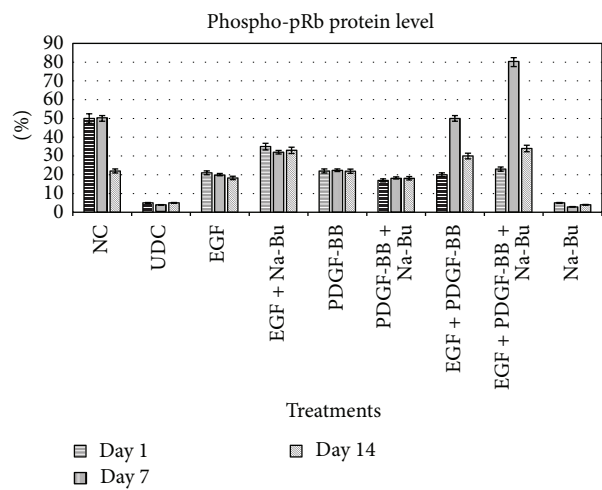


FIGURE 6: Effect of various treatments on the percentage of phosphorylated pRb protein. The various treatments are given in the x-axis. The percentage of pRb is given in y-axis with Standard Mean Error. The horizontal line is day 1, vertical line is day 7, and diagonal line is day 14 after the wounding when the tissue was collected. NC, normal control; UDC, untreated diabetic control; the growth factor and Na-Bu treatments are as given below each panel.

the individual growth factor application. Approximately 20 to 35% of the total pRb was found in phosphorylated form with the individual growth factors plus Na-Bu combination. The combination of EGF plus PDGF-BB resulted in a 4- to 5-fold increase in total pRb protein and 30 to 50% of it existed in phosphorylated form. The combination of EGF plus PDGF-BB plus Na-Bu increased the level of total pRb by approximately 6- to 7-fold on days 7 and 14 after treatment. More importantly with this combination approximately 80%

of total pRb remained in phosphorylated form on day 7. On day 14, approximately 70% of total pRb existed in hypo-phosphorylated form and it was statistically significant. It has to be noted that the Na-Bu treated wound had less than 5% of pRb in phosphorylated form (Figures 1 and 6).

#### 4. Discussion

The process of wound healing requires a highly regulated mechanism at the molecular level and may be divided into three phases: (i) inflammation, (ii) cell proliferation, and (iii) cell differentiation/maturation [18, 19]. Topical application of EGF plus PDGF-BB produced the best wound healing in acute cutaneous wound healing model in mice [3–5]. These growth factors enhanced wound repair process through increased expression of their corresponding receptor mRNAs, proteins, and receptor protein phosphorylation [4, 5]. Delayed or defective wound healing causes a significant morbidity in diabetic patients [20, 21]. Reduced levels of a number of growth factors including PDGFs and EGF have been shown in the chronic wound as compared to the acute wound [22] and it is proposed that destruction of growth factors by proinflammatory cytokines is responsible for such reduction [23]. Therefore, the effect of topically applied growth factors was tested by many groups in chronic as well as diabetic wound healing, some of which produced promising results. For example, topical application of EGF helped diabetic wound healing in experimental animal model [7] and in patients with foot ulcers [6]. Application of PDGF improved experimental diabetic wound healing [8, 9]. Both EGF and PDGFs aided tissue granulation at the wound site [10, 24]. Data from the present study shows that daily topical application of growth factors EGF and PDGF-BB at the immediate early stages, either individually or in combination, partially helped the diabetic wound healing process. This is in agreement with previous studies where these growth factors have shown to be beneficial in the diabetic wound healing in human and animal models [6–10].

The reduced levels of EGF-R and  $\beta$ -PDGF-R in the untreated diabetic wound and its concomitant increase in the growth factor treated wound (Figures 1, 2, and 3) indicate that such an increase could have partially aided the diabetic wound healing (Table 4). The reduced efficacy of EGF plus Na-Bu is due to drastic reduction in the levels of EGF-R and  $\beta$ -PDGF-R (Figures 1, 2, and 3) as these proteins are necessary for cell migration and proliferation during wound healing process [25, 26]. Previous report showed no synergy between PDGF-BB and EGF in diabetic wound healing in mice [27]. Our data confirms this report as this combination did not improve the diabetic wound closure and failed to produce additive increase in receptor protein levels (Figures 1, 2, and 3). It has been shown that Na-Bu treatment leads to withdrawal of cells from cell cycle [28] and the current data confirms this report as Na-Bu caused less than 5% wound closure (Table 4) perhaps due to withdrawal of most cells from cell cycle at the wound site.

Sodium butyrate induces cell differentiation [13] and inhibits HDAC1 [14, 15]. HDAC have essential, pleotropic roles in cell proliferation and HDAC1 prevents expression of

antiproliferative genes in cycling cells [29]. HDAC1 has a regulatory role in stem cell renewal by maintaining expression of key pluripotent transcription factors [30]. HDAC is essential for unrestricted cell proliferation by repressing selective cell cycle inhibitors and HDAC1 represses the CDK inhibitor p21 [29]. The partial wound healing in diabetic mice caused by the application of EGF and PDGF-BB appears to be through increased expression of HDAC1 (Figures 1 and 4) and decreased p21 protein expression (Figure 5). The p21 protein is an inhibitor of CDK2/cyclin complex and CDK2/cyclin (cyclins D and E) complexes phosphorylate pRb protein. Phospho-pRb is necessary for G1 to S-phase transition of cells during cell cycle [31]. Thus, it appears that higher level of HDAC1, drastic downregulation of p21 protein, and increased level of total pRb with increased percentage of phosphorylated pRb are necessary for efficient cell proliferation at the initial stages of wound healing in diabetic cases (Figures 1, 4, 5, and 6). Increased level of total pRb and increased percentage of hypophosphorylated form of pRb were reported in Na-Bu induced differentiation in cultured human colon cancer cell line HT29 [13]. Our data shows that increased percentage of hypophosphorylated pRb in the later stages of wound healing could be necessary for cell differentiation in diabetic cases (Figures 1 and 6).

Exudate from nonhealing diabetic wound had numerous mediators characteristic for persistent inflammatory and tissue destructive response as compared to the healing wound which had many differentiation markers [32]. High levels of serine protease inhibitor, SerpinB3, were reported as a biomarker for successful wound healing in diabetic patients [33]. Erythropoietin is a novel repurposed drug for diabetic wound healing which promotes cell differentiation [34]. Thus, it appears that, after initial growth factor application that promotes cell proliferation, addition of a cell differentiation factor, Na-Bu, is essential at later stages for normal diabetic wound healing. In addition to degradation of growth factors, the proinflammatory cytokines [23] could also degrade differentiation factors thereby preventing or delaying cellular differentiation. Growth factors-induced cell proliferation when not accompanied by cell differentiation could perhaps lead to exhaustion of the number of dividing cells which in turn could lead to unhealed or partially healed diabetic wound.

Therefore, addition of exogenous differentiation agent(s) might also be necessary for efficient diabetic wound healing process. However, our data indicates that the exogenous applications of cell proliferation agents such as growth factors and cell differentiation agents such as Na-Bu should be done in a sequential, sustained, and time-bound manner (Table 4). The application of growth factors should be performed immediately after wounding in order to facilitate cell migration to the wound site and cell proliferation and it should precede the application of Na-Bu (Table 4). Once the growth factors set the cell proliferation process in motion, the newly divided cells need to be differentiated for effective wound closure. Therefore, the differentiation agent should be provided at a little later stages in order to achieve maximum benefit during diabetic wound healing process. Addition of differentiation agent such as Na-Bu alone at the immediate early stages of the

wound healing process is deleterious for the wound healing process (Table 4; Figure 1) because Na-Bu could prevent cell proliferation [35] and induce apoptosis as observed in cultured cells [36].

The concentration of Na-Bu used in the present study is 2 mM which is half of that of its physiological concentration of 5 mM [37]. The subphysiological concentration of 2 mM Na-Bu used in our study could aid cell differentiation and perhaps could not totally block the growth factor aided cell cycle to proceed so as to accomplish normal healing of diabetic wound. Topical application of growth factors and/or Na-Bu did not affect the blood glucose level in the treated mice as compared to the untreated ones. These applications did not cause any side effects either at the wound site or to the whole animal (unpublished observation).

## 5. Conclusions

Currently the use of recombinant human PDGF-BB and Becaplermin gel as an adjuvant therapy is approved by the Food and Drug Administration to treat diabetic wound [38]. It is sold with a commercial name "REGRANEX" with a warning that "people who use 3 or more tubes of REGRANEX Gel may have increased risk of death from cancer" (<http://www.regranex.com/patient/>). The results from our experiments suggest that addition of a cell differentiation agent, Na-Bu, could counter such carcinogenic side effects of PDGF-BB and also EGF. Sodium butyrate also could minimise the carcinogenic effect of PDGF-BB in the commercially available REGRANEX gel if a diabetic patient has to use more than 2 tubes to treat the wound. It is conceivable that only cell proliferation is happening in some of these diabetic wounds treated with REGRANEX with minimal or no cell differentiation. Therefore, addition of sodium butyrate in the 3rd or even in the 2nd tube of REGRANEX gel could help maximise cell differentiation in addition to cell proliferation leading to effective diabetic wound healing. Such an addition of sodium butyrate could also minimise or reduce the potential carcinogenic effect of PDGF-BB. It could do so because sodium butyrate is an anti-cell proliferative agent and it is also known to induce apoptotic cell death in cultured cancer cells [35, 36]. Thus, the topical application of combination of EGF plus PDGF-BB along with a differentiation inducing agent (Na-Bu) may eventually be considered for clinical treatment and management of various human wounds and burns.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Rohini Keshava and Rajalakshmi Gope made equal contribution.

## Acknowledgments

The authors thank Dr. J. Sureshchandra, Senior Veterinary Officer, and Mr. K. S. Yogeesh, Central Animal Research



Facility, NIMHANS, Bangalore, for their kind cooperation. The authors also thank Dr. D. K. Subbakrishna for his help with statistical data analysis and NIMHANS for providing infrastructure and other support to carry out the experiments. Rohini Keshava is a Postdoctoral Research Fellow of Kothari foundation (UGC).

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