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Research Article
L-Glutamine Accelerates Wound Healing in Diabetic Foot Ulcers in Experimental Rats

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Abstract
Background and Objective: Diabetes mellitus is a devastating condition and an important risk factor for the delayed healing process, leading to chronic Diabetic Foot Ulcers (DFUs). L-glutamine showed its antidiabetic and wound healing potential against various experimental models. To evaluate the potential of L-glutamine against the STZ-induced excision wound model, which mimics the clinical characteristic of DFU. **Materials and Methods:** Wounds (2×5 mm) were created on the dorsal surface of the paw of diabetic Sprague Dawley rats and then they were treated with either vehicle or L-Glutamine (250, 500 and 1000 mg kg^{-1}, p.o.) or insulin (10 IU mg kg^{-1}, s.c.) for 21 days. A group of non-diabetic and normal wound control animals was maintained separately. **Results:** L-Glutamine (500 and 1000 mg kg^{-1}) treatment effectively ($p<0.05$) accelerates wound healing revealed by an increased rate of wound contraction and decreased wound area compared to control group animals. Diabetes-induced elevated oxido-nitrosative stress (SOD, GSH, MDA and NO) and proinflammatory release (TNF-α and IL-1β) were markedly ($p<0.05$) attenuated by L-Glutamine. Down-regulation production of hydroxyproline and mRNA expressions of Nrf2 and Ang-1in wound tissues was effectively ($p<0.05$) increased by L-Glutamine. It also significantly attenuated ($p<0.05$) diabetes-induced alteration in wound architecture. **Conclusion:** L-glutamine improve the wound healing process via accelerated angiogenesis and inhibition of hyperglycemia, oxidative stress and release of inflammatory mediators in experimental diabetic foot ulcer.

Key words: Ang-1, Diabetic Foot Ulcer, L-Glutamine, Nrf2, TNF-α, angiogenesis, hyperglycemia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Healing is the indispensable part of human evolution where destruction of the functionality of living tissue continuity due to various physical and chemical agents results in the formation of wounds[1]. The restoration of the structural architecture of damaged tissue is a highly dynamic and complex process. This involved physiological and biochemical modification towards the repair and regeneration of tissue structure known as wound healing[2,3]. During normal conditions, wound healing occurs much faster, however, certain conditions, including age, microbial infection, elevated blood sugar levels, poor blood circulation, oedema, poor nutrition, etc., interfere in the various phases of wound healing, thus impairing tissue healing[4,5]. Diabetes mellitus is a devastating condition and an important risk factor for the delayed healing process, leading to chronic Diabetic Foot Ulcers (DFUs)[6-8]. It has been estimated that almost 12% of patients with diabetes are prone to develop DFUs, amongst which approximately 84% of patients needed amputations of the lower leg due to non-healing DFU[6]. DFU contributes to amputation and is also associated with significant healthcare resource utilization costs and worsened quality of life. A recent economic burden study reported the cost for management of DFU is approximately US$1960[7]. Moreover, according to the EpiCast report, the cases of DFU have been expected to reach approximately 1.5 million by 2025[9].

It has been well documented that Diabetes Mellitus (DM) is one of the important devastating risk factors that interfere in the wound healing process[10,11]. Hyperglycemia, impaired carbohydrate metabolism, altered insulin production and glycosuria have been suggested as vital DM-associated long-term complications that interact and modify microvasculature, thus further contributing to DFU[12,13]. Furthermore, elevated generation of reactive nitrogen species and reactive oxygen species, including hydroxyl radical, hydrogen peroxide and superoxide, activates inflammatory response and induces oxidative stress that plays a significant role in the pathogenesis of DFU[10,14,15]. Researchers suggested that DFU is mainly characterized by the aggravated response of neutrophil granulocytes at the wound site, which stimulates proinflammatory cytokines including TNF-α (Tumour Necrosis Factor-α) and IL-1β (Interleukin-1 Beta)[15-17]. These cytokines cause the degradation of the tissue protein matrix and inhibit the production of growth factors via the formation of MMP (Matrix Metalloprotease), thereby regulating the normal wound healing process. Therefore, most researchers have targeted inhibition of inflammatory and oxidative pathways to accelerate the angiogenesis process during wound healing in DM patients[18,19].

L-glutamine, a non-essential amino acid that is an important precursor for glutamine synthesis, thus serves as an important source of natural antioxidants[20,21]. Researchers have utilized its glutamine augmenting potential against various diseases, including inflammation, diabetes, ulcer, cancer, cardiotoxicity, hepatotoxicity and nephrotoxicity[20,22-24]. Sadar *et al.*[25] documented the nephroprotective role of L-glutamine via inhibition of elevated oxidative stress and Transforming Growth Factor-β (TGF-β) during diabetic nephropathy[25]. Clinical studies have documented its safety after oral administration in normal and diseased volunteers[26]. Furthermore, the protective potential of L-glutamine has been well documented in patients with catabolic disorders, patients with intestinal toxicity[21] and catabolic surgical patients[27]. Furthermore, several researchers have well-documented wound healing potential of L-glutamine against an experimental animal model of burn wound[28], incision wound[29] and excision wound model[30]. Treatment of L-glutamine in burned patients and the postoperative patient showed the increased potential of neutrophils to destroy bacteria, further decreasing postoperative events and number of days in the hospital[31,32]. However, its potential against DFU has not been documented yet. Hence, the present investigation aimed to evaluate the potential of L-glutamine against the STZ-induced excision wound model, which mimics the clinical characteristic of DFU.

MATERIALS AND METHODS

Study area: The experiment was performed in Xi'an Jiaotong University, China, from 4th November, 2020 and 5th April, 2021. All the experiments were carried out between 08:00 and 17:00 hrs in a quiet laboratory environment.

Animals: Adult Sprague Dawley rats (male, 180-220 g, n = 110) were obtained from The Fourth People's Hospital of Shaanxi. Rats were housed at 24±1°C, with a relative humidity of 45-55% and a 12:12 hrs dark/light cycle. The animals had free access to standard pellet chow and filtered water throughout the experimental protocol. Experiments were conducted during 09:00-17:00 hrs. The experimental protocol (approval number 20210791) was approved by The Fourth People's Hospital of Shaanxi and performed following the guidelines of the National Institute of Health Guide for Care and Use of Laboratory Animals.

Chemicals: L-Glutamine and STZ (Sigma-Aldrich), Crystalline beef liver catalase, 1,1,3,3-tetraethoxypropane, 5,5'-dithiobis

Table 1: Primer sequences for Ang-1, Nrf2, TNF-α, IL-1β and β-actin

Gene	Primer sequence Forward primer	 Reverse primer	Size (bp)
Ang-1	GCTGGCAGTACAATGACAGT	GTATCTGGGCCATCTCCGAC	512
Nrf2	CCTCACCTCTGCTGCCAGT	GGGAGGAATTCTCCGGTCTC	316
TNF-α	AAGCCTGTAGCCCATGTTGT	CAGATAGATGGGCTCATACC	295
IL-1β	TGATGTTCCCATTCHACAGC	GAGGTGCTGATGTACCAGTT	378
β-actin	GTCACCCACACTGTGCCCATCT	ACAGAGTACTTGCGCTCAGGAG	764

(2-nitrobenzoic acid) and reduced glutathione (SD Fine Chemicals, Mumbai, India), Naphthalene-2,3-diamine hydrochloride, sulphanilamide and phosphoric acid (Loba Chemie Pvt. Ltd.), Insulin injection (Novo Nordisk, India), One-step RT-PCR and Total RNA Extraction kit (MP Biomedicals India Private Limited, India) were procured from respective suppliers.

Induction and assessment of diabetes: Streptozotocin (STZ) was dissolved in citrate buffer (0.1 M, pH 4.4) and administered intraperitoneally at a 55 mg kg^{-1} dose to induce diabetes in rats[33]. A separate group of age matched control rats was also maintained, which received an equal volume of citrate buffer. Rats with serum glucose levels of more than 250 mg dL^{-1} were selected as diabetic and used for the present study.

Excision wound model: According to a previously established method, an excision diabetic foot ulcer was created in anaesthetized rats (ketamine (75 mg kg^{-1}, i.p.) and xylazine (10 mg kg^{-1}, i.p.))[18]. Then, the rectangular wound (2×5 mm) was created on the dorsal surface of the foot of each rat. The excision-wound-created rats were assigned to various treatment groups randomly, as mentioned in the experimental design.

Experimental design: After wound creation, rats were divided into the various groups (n = 8-10, each) viz., normal non-diabetic (ND, without wound, received double distilled water (DW, 10 mg kg^{-1}, p.o.)), normal wound control (NWC, non-diabetic animals with wounds received DW (10 mg kg^{-1}, p.o.), Diabetic Wound Control (DWC) animals either received (DW (10 mg kg^{-1}, p.o.) or treated with L-Glutamine (LG, 250, 500 and 1000 mg kg^{-1}, p.o.) or insulin (10 IU mg kg^{-1}, s.c.) for 21 days. On different days, an observer blind to the treatment used a digital camera (Fuji, S20 Pro, Japan) to capture the photos to determine the wound area. The metabolic cages (Techniplast, Italy) were used to determine food intake and water intake. The recording of Motor Nerve Conduction Velocity (MNCV) was measured according to a previously reported method[34].

After 22 days, rats were anaesthetized under ethereal anaesthesia. Blood was withdrawn by a retro-orbital puncture and serum was separated by centrifugation (4°C, 5200 g, 15 min) using Eppendorf Cryocentrifuge (model No. 5810, Germany) to determine serum insulin by a rat ELISA (Enzyme-Linked Immunosorbent Assay) kit (Mercodia AB, Uppsala, Sweden). The cervical dislocation method was used to sacrifice these animals. Then wound tissues were immediately isolated and maintained at -80°C for further biochemical analysis. The Superoxide Dismutase (SOD), reduced Glutathione (GSH), lipid peroxidation (MDA), Nitric Oxide (NO) and hydroxyproline content was determined in wound tissue as described previously[34]. The quantitative real-time (qRT)-PCR of Ang-1, Nrf2, TNF-α and IL-1β was used to determine mRNA levels in skin tissue[34]. The primer sequence for Ang-1, Nrf2, TNF-α and IL-1β is provided in Table 1. Another portion of the skin was cut into 3-5 μm thickness sections and stained with hematoxylin and eosin (H and E) for various histological features.

Statistical analysis: Data are expressed as Mean±Standard Error Mean (SEM). GraphPad Prism 5.0 software (GraphPad, San Diego, CA) was used to achieve data analysis. Data of body weight, serum glucose level, wound area and percent wound closure was analyzed by two-way ANOVA followed by Tukey's multiple range post hoc analysis, whereas biochemical and molecular parameters were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc analysis, Kruskal-Wallis test was used as a post hoc analysis to analyzed non-parametric tests. A p<0.05 was measured to be statistically significant.

RESULTS

Effect of L-Glutamine treatment on body weight, serum glucose level, rate of wound contraction and CT50 of diabetic rats: Induction of diabetes caused delayed wound healing reflected by aggravated wound area in DWC rats. However, normal wound control rats showed expedited wound healing depicted by significant (p<0.05) decreased wound area and increased wound contraction rate of wound

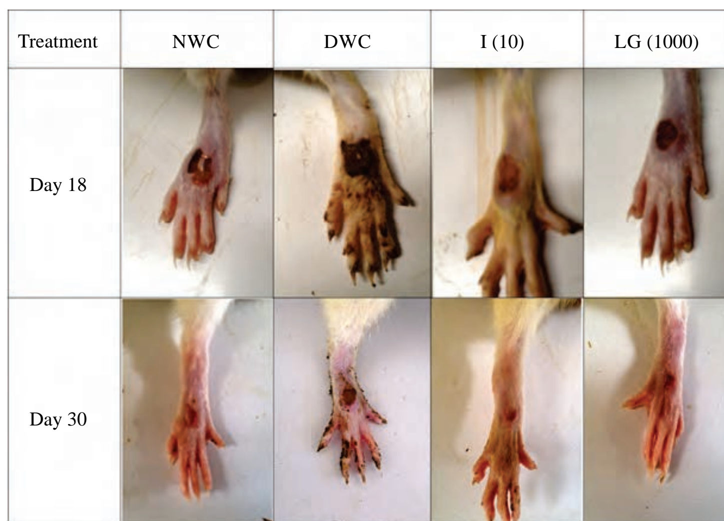

Fig. 1: Effect of L-Glutamine treatment wound healing
Morphological representation of rat paws showing various phases of wound healing

Table 2: Effect of L-Glutamine treatment on food intake, water intake, urine output, serum insulin, CT50 and Motor nerve conduction velocity of diabetic rats

Treatment	Food intake (gm)	Water intake (mL)	Urine output (mL)	Serum insulin (mg L^{-1})	CT_{50} (days)	Motor nerve conduction velocity (m/s)
Normal ND	18.25±0.53	63.83±2.04	16.17±1.35	2.60±0.09	-	59.12±1.87
NWC	19.57±0.70	67.00±2.31	18.83±1.38	2.44±0.09	23.68	55.54±2.11
DWC	41.47±1.01$^{\#}$	124.20±1.83$^{\#}$	58.83±0.91$^{\#}$	0.99±0.09$^{\#}$	-54.27	26.66±2.25$^{\#}$
I (10)	23.77±0.84$^{*,\$}$	72.83±2.74$^{*,\$}$	22.50±1.46$^{*,\$}$	2.41±0.07$^{*,\$}$	28.74	44.67±0.48$^{*,\$}$
LG (250)	37.73±0.73	120.00±1.65	56.17±1.20	1.17±0.09	74.14	29.53±2.70
LG (500)	30.83±0.95$^{*,\$}$	97.17±2.26$^{*,\$}$	45.00±0.93$^{*,\$}$	1.75±0.07$^{*,\$}$	40.32	42.52±1.43$^{*,\$}$
LG (1000)	26.22±0.83$^{*,\$}$	82.33±2.65$^{*,\$}$	29.50±0.89$^{*,\$}$	2.18±0.06$^{*,\$}$	31.07	48.60±0.65$^{*,\$}$

Data are expressed as mean±SEM (n = 6) and analyzed by one-way ANOVA followed by Tukey's multiple range test for each parameter separately. $^{\&}$p<0.05 as compared to normal non-diabetic, $^{\#}$p<0.05 as compared to normal wound control, *p<0.05 as compared to the diabetic wound control group and $^{\$}$p<0.05as compared to one another. ND: Non-diabetic, NWC: Normal wound control group, DWC: Diabetic wound control group, I (10): Insulin (10 IU kg^{-1}, s.c.) treated group, LG (250): L-Glutamine (250 mg kg^{-1}, p.o.) treated group, LG (500): L-Glutamine (500 mg kg^{-1}, p.o.) treated group, LG (1000): L-Glutamine (1000 mg kg^{-1}, p.o.) treated group and CT50: Time at which 50% of the cutaneous wound was closed

closure compared to DWC rats. Administration of insulin and L-Glutamine (500 and 1000 mg kg^{-1}) also showed a noticeable (p<0.05) decreased wound area and increased wound contraction rate or wound closure as compared to DWC rats (Fig. 1 and 2a,b).

The body weight of DWC rats was gradually decreased (p<0.05) over 30 days as compared to non-diabetic rats, whereas DWC rats exhibited a marked increase (p<0.05) in serum glucose levels as compared to non-diabetic rats. Administration of insulin and L-Glutamine (500 and 1000 mg kg^{-1}) showed an effective inhibition (p<0.05) in STZ-induced alteration body weight and serum glucose levels as compared to DWC rats (Fig. 2c-d).

Effect of L-Glutamine treatment on food intake, water intake, urine output and serum insulin of diabetic rats: There was no significant difference in food intake, water intake, urine output and serum insulin levels in DWC rats compared to non-diabetic rats. However, food intake, water intake and urine output were elevated (p<0.05), whereas serum insulin levels were decreased in DWC rats compared to non-diabetic rats. Chronic treatment with insulin strikingly repressed (p<0.05) increased food intake, water intake and urine output as well as it effectively (p<0.05) increased serum insulin levels as compared to DWC rats. Conversely, administration of L-Glutamine (500 and 1000 mg kg^{-1}) also showed a decreased food intake, water intake and urine output and increased serum insulin levels compared to DWC rats. However, insulin treatment showed more effective (p<0.05) attenuation in elevated food intake, water intake and urine output as compared to L-Glutamine treatment (Table 2).

Effect of L-Glutamine treatment on motor nerve conduction velocity of diabetic rats: When compared with non-diabetic

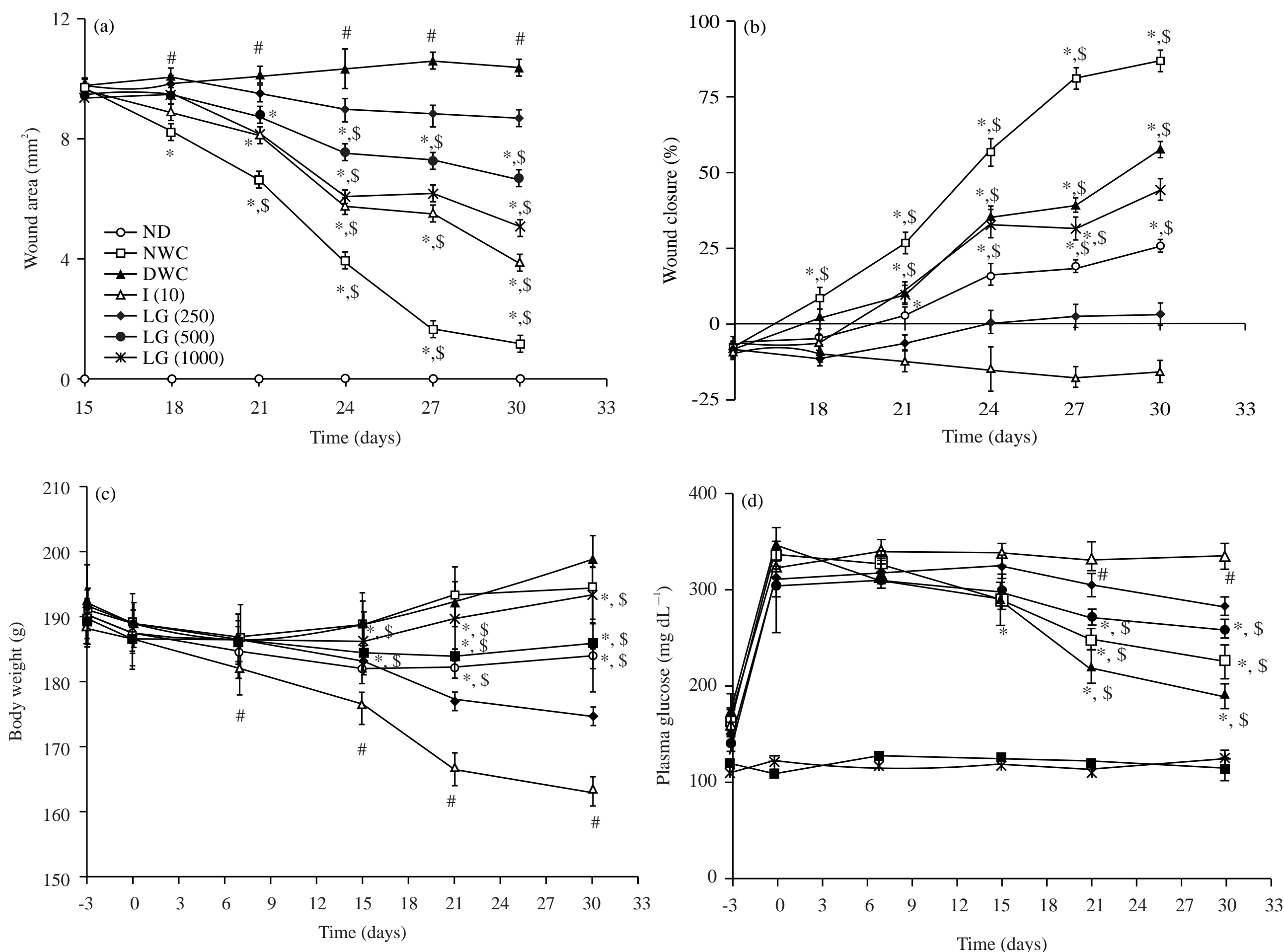

Fig. 2(a-d): Effect of L-Glutamine treatment on wound area (a), percent wound closure (b), body weight (c) and serum glucose level (d) in diabetic rats

Data are expressed as Mean±SEM (n = 6) and analyzed by two-way ANOVA followed by Tukey's multiple range test for each parameter separately, &p<0.05 as compared to normal non-diabetic, #p<0.05 as compared to normal wound control, *p<0.05 as compared to the diabetic wound control group and $p<0.05 as compared to one another

rats, MNCV was decreased significantly (p<0.05) in DWC rats. Administration of insulin effectively (p<0.05) attenuated diabetes-induced decreased MNCV as compared to DWC rats. L-Glutamine (500 and 1000 mg kg^{-1}) treatment also showed an effective increase (p<0.05) in MNCV as compared to DWC rats. Inhibition of STZ induced decreased MNCV was more prominent in L-Glutamine (1000 mg kg^{-1}) than insulin treatment (Table 2).

Effect of L-Glutamine treatment on the oxido-nitrosative stress in the wound of diabetic rats: Induction of wound caused a significant increase (p<0.05) in oxido-nitrosative stress in NWC control rats as compared to normal non-diabetic rats and this oxido-nitrosative stress more prominently aggravated (p<0.05) in DWC rats due to induction of diabetes as compared to NWC. DWC rats showed a noticeable (p<0.05) decrease in SOD and GSH levels and increased MDA and NO levels than NWC control rats. Insulin treatment showed significant (p<0.05) attenuation in oxido-nitrosative stress compared to DWC rats. Treatment with L-Glutamine (500 and 1000 mg kg^{-1}) showed (p<0.05) marked increase in SOD and GSH whereas a decrease in MDA and NO levels as compared to DWC rats (Table 3).

Effect of L-Glutamine treatment on hydroxyproline level of diabetic rats: DWC rats exhibited a marked (p<0.05) decrease in hydroxyproline level in wound tissue than non-diabetic rats. Additionally, NWC rats also showed significantly (p<0.05) decreased hydroxyproline levels than non-diabetic rats. However, administration of insulin

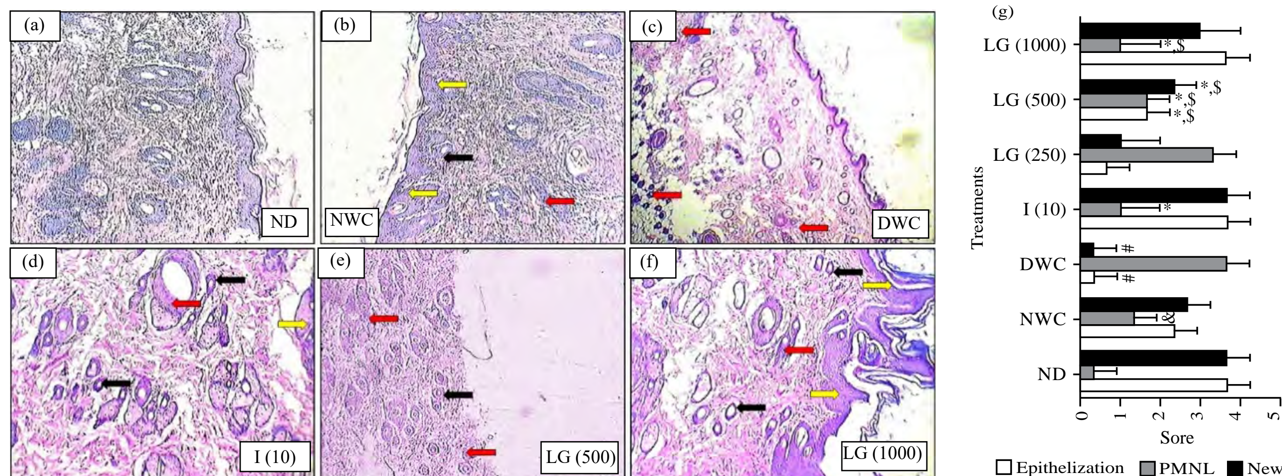

Fig. 3(a-g): Effect of L-Glutamine treatment on wound histology in diabetic rats

Photomicrographs of sections of wound skin from normal non-diabetic (A), normal wound control (B), diabetic (STZ) wound control (C), insulin (10 IU kg^{-1}, s.c.) (D), L-Glutamine (500 mg kg-1, p.o.) (E) and L-Glutamine (1000 mg kg^{-1}, p.o.) (F). The quantitative analysis of the Effect of L-Glutamine treatment on wound histology (G). Data are expressed as Mean±SEM (n = 3) and analyzed by one-way ANOVA followed by the Kruskal-Wallis test for each parameter separately. $^{\&}$p<0.05 as compared to normal non-diabetic, $^{\#}$p<0.05 as compared to normal wound control, *p<0.05 as compared to the diabetic wound control group and $^{\$}$p<0.05 as compared to one another. Hematoxylin and eosin-stained staining at 100 X. Epithelialization (yellow arrow), New vessel formation (black arrow) and Polymorphonuclear neutrophil leukocytes infiltration (red arrow)

Table 3: Effect of L-Glutamine treatment on the oxide-nitrosative stress and hydroxyproline level in the wound of diabetic rats

Treatment	SOD (U mg^{-1} of protein)	GSH (µg mg^{-1} of protein)	MDA (nM mg^{-1} of protein)	NO (µg mL^{-1})	Hydroxyproline (µg mg^{-1} tissue)
Normal ND	3.02±0.12	14.07±0.47	4.34±0.62	74.67±5.67	4.09±0.24
NWC	2.30±0.16$^{\&}$	10.33±0.76$^{\&}$	9.04±0.13$^{\&}$	131.60±4.61$^{\&}$	3.14±0.19$^{\&}$
DWC	1.38±0.16$^{\#}$	5.69±0.47$^{\#}$	24.24±0.51$^{\#}$	260.90±1.66$^{\#}$	1.94±0.15$^{\#}$
I (10)	2.79±0.12$^{*,\$}$	13.01±0.61$^{*,\$}$	10.78±0.48$^{*,\$}$	133.00±5.41$^{*,\$}$	3.96±0.17$^{*,\$}$
LG (250)	1.68±0.16	4.96±0.52	21.67±0.27	251.30±3.80	2.08±0.08
LG (500)	2.22±0.13$^{*,\$}$	9.76±0.60$^{*,\$}$	15.87±0.39$^{*,\$}$	183.20±3.44$^{*,\$}$	2.95±0.11$^{*,\$}$
LG (1000)	2.29±0.09$^{*,\$}$	10.61±0.77$^{*,\$}$	10.82±0.54$^{*,\$}$	155.70±4.39$^{*,\$}$	3.48±0.21$^{*,\$}$

Data are expressed as mean±SEM (n = 6) and analyzed by one-way ANOVA followed by Tukey's multiple range test for each parameter separately. $^{\&}$p<0.05 as compared to normal non-diabetic, $^{\#}$p<0.05 as compared to normal wound control, *p<0.05 as compared to the diabetic wound control group and $^{\$}$p<0.05 as compared to one another. ND: Non-diabetic, NWC: Normal wound control group, DWC: Diabetic wound control group, I (10): Insulin (10 IU kg^{-1}, s.c.) treated group, LG (250): L-Glutamine (250 mg kg^{-1}, p.o.) treated group, LG (500): L-Glutamine (500 mg kg^{-1}, p.o.) treated group, LG (1000): L-Glutamine (1000 mg kg^{-1}, p.o.) treated group, SOD: Superoxide Dismutase, GSH: Reduced Glutathione, MDA: Malondialdehyde and NO: Nitric Oxide

effectively (p<0.05) increased hydroxyproline level compared to DWC rats. L-Glutamine (500 and 1000 mg kg^{-1}) treatment also notably (p<0.05) inhibited diabetes-induced decreased hydroxyproline level in wound tissue of rats (Table 3).

Effect of L-Glutamine treatment on Ang-1, Nrf2, TNF-α and IL-1β mRNA expressions in wound skin tissue of diabetic rats: The mRNA expressions of Ang-1 and Nrf2 were markedly (p<0.05) down-regulated in NWC and more prominently (p<0.05) down-regulated DWC rats as compared to non-diabetic rats. Whereas TNF-α and IL-1β mRNA expressions were significantly (p<0.05) up-regulated in NWC and DWC rats than non-diabetic rats. Administration of insulin noticeably (p<0.05) up-regulated Ang-1 and Nrf2 mRNA expressions whereas down-regulated TNF-α and IL-1β mRNA expressions compared to DWC rats. L-Glutamine (500 and 1000 mg kg^{-1}) treatment also showed a significant (p<0.05) inhibition in diabetes-induced alteration in Ang-1, Nrf2, TNF-α and IL-1β mRNA expressions in wound tissue of rats when compared with DWC rats. L-Glutamine (1000 mg kg^{-1}) treatment showed more effective up-regulation in Ang-1 mRNA expression than insulin treatment (Table 4).

Effect of L-Glutamine treatment on diabetes-induced histopathological alterations in skin tissue of diabetic rats: In Fig. 3a represented normal skin tissue architecture with the

Table 4: Effect of L-Glutamine treatment on the Ang-1, Nrf2, TNF-α and IL-1β mRNA expression in wound of diabetic rats

Treatment	Ang-1/β-actin	Nrf2/β-actin	TNF-α/β-actin	IL-1β/β-actin
Normal ND	3.42±0.03	2.95±0.15	0.51±0.12	0.94±0.07
NWC	1.73±0.17$^{\&}$	1.54±0.08$^{\&}$	1.19±0.11$^{\&}$	1.31±0.16$^{\&}$
DWC	0.45±0.13$^{\#}$	0.88±0.15$^{\#}$	2.08±0.15$^{\#}$	2.03±0.08$^{\#}$
I (10)	2.35±0.10$^{*,\$}$	2.41±0.13$^{*,\$}$	0.89±0.12$^{*,\$}$	1.13±0.08$^{*,\$}$
LG (250)	0.59±0.09	1.09±0.08	2.10±0.12	1.99±0.19
LG (500)	1.78±0.17$^{*,\$}$	1.79±0.13$^{*,\$}$	1.60±0.10$^{*,\$}$	1.74±0.12$^{*,\$}$
LG (1000)	2.55±0.14$^{*,\$}$	2.19±0.13$^{*,\$}$	1.11±0.17$^{*,\$}$	1.32±0.14$^{*,\$}$

Data are expressed as mean±SEM (n = 4) and analyzed by one-way ANOVA followed by Tukey's multiple range test for each parameter separately. $^{\&}$p<0.05 as compared to normal non-diabetic, $^{\#}$p<0.05 as compared to normal wound control, *p<0.05 as compared to the diabetic wound control group and $^{\$}$p<0.05 as compared to one another. ND: Non-diabetic, NWC: Normal wound control group, DWC: Diabetic wound control group, I (10): Insulin (10 IU kg^{-1}, s.c.) treated group, LG (250): L-Glutamine (250 mg kg^{-1}, p.o.) treated group, LG (500): L-Glutamine (500 mg kg^{-1}, p.o.) treated group, LG (1000): L-Glutamine (1000 mg kg^{-1}, p.o.) treated group, Ang-1: angiopoietin-1, Nrf2: nuclear factor erythroid 2–related factor 2, TNF-α: tumour necrosis factor-α and IL-1β: Interleukin-1 Beta

presence of well-organized dermis and epidermis, epithelial layer and blood vessels with mild Polymorphonuclear Neutrophil Leukocytes (PMNL) infiltration. Induction of wound caused significant (p<0.05) increase infiltration of PMNL cells into wound tissue reflected in the histology of skin from NWC rats (Fig. 3b). Moreover, induction of diabetes caused delayed wound healing reflected by a slow progression in the formation of epithelial layer and new blood vessels along with significant (p<0.05) presence of PMNL (Fig. 3c). However, administration of insulin and L-Glutamine (500 and 1000 mg kg^{-1}) inhibited diabetes-induced delayed wound healing depicted by a significant (p<0.05) decreased in PMNL infiltration and increased formation of the epithelial layer and new blood vessels as compared to DWC rats (Fig. 3d-g), respectively.

DISCUSSION

Wound healing is a simple linear process that involves the interaction of various biological molecules and cells towards the wound contraction and re-epithelialization[35]. However, certain predisposing conditions such as diabetes mellitus interfere with the wound healing process, resulting in more chronic and complex ulcer conditions that affect the prognosis of the disease[4]. Literature lucid with evidence suggested that L-glutamine contains wound healing, antibacterial, anti-inflammatory and antidiabetic potential, which increased its attention towards the treatment of DFU. In the present investigation, we have determined the wound healing potential of L-glutamine against experimental DFU. The findings suggested that L-glutamine improves the wound healing process in the diabetic condition through accelerated angiogenesis (Ang-1) and amelioration of elevated blood glucose levels, oxidative stress (Nrf2, SOD, GSH, MDA and NO) and inflammatory release (TNF-α and IL-1β).

In the present investigation, administration of L-glutamine significantly ameliorated diabetes-induced elevated blood glucose, decreased insulin and neuropathic abnormalities, which may accelerate wound healing. Insulin plays a vital role in the pathophysiology of delayed wound healing[14,36]. Elevated production of free fatty acid cause insulin resistance, which further inhibits glucose transport and leads to hyperglycaemia[14,37]. It has been well documented that chronic hyperglycemia is responsible for various tissue damage, resulting in diabetic complications, including cardiomyopathy, neuropathy, retinopathy and foot ulcer[38,39]. Thus, insulin resistance is one of the important pathophysiological failures of many pre-diabetic conditions and results in metabolic failure[40]. Furthermore, elevated serum glucose levels are responsible for modifying microvascular permeability and Motor Nerve Conduction Velocity (MNCV)[41]. A combination of these conditions leads to neuropathic abnormalities in the feet of DM patients and this situation increases the risk of chronic infections, which result in DFU[10,42]. Thus, elevated blood glucose levels and impaired MNCV have a direct relation with DFU. The previous investigator also suggested that L-glutamine inhibited diabetic complications via inhibition of hyperglycemia[25] and the findings of the present study are in line with this researcher.

Treatment with L-glutamine inhibited STZ-induced elevated oxido-nitrosative stress, contributing to accelerated wound healing. Clinically, it has been proven that elevated free radicals caused induction of oxidative stress, resulting in progressive worsening of the wound healing process[43,44]. Thus, the down-regulation of the elevated response of ROS has been shown a beneficial effect in diabetic patients and associated complications, including DFU[6,45]. Researchers documented that STZ-induces an increase in ROS release, which further depletes the intracellular levels of SOD, a superoxide scavenger and augmentation of lipid peroxidation

reflected by elevated levels of MDA[46]. Thus, an imbalance between intracellular antioxidant enzymes (SOD and GSH) and ROS production plays a vital role in worsening the wound healing process[47,48]. Furthermore, elevated Nitric Oxide (NO) quickly reacts with superoxide anions and leads to peroxynitrite formation, further aggravating lipid peroxidation[49]. Numerous pieces of evidence suggested that Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor responsible for activating antioxidant enzymes including SOD, catalase, GSH and glutathione peroxidase[15,50,51]. Tissue injury causes activation of Nrf2, which further synergizes with other transcription, thus promoting wound healing[52]. Previously, various researchers documented the Nrf2-activation potential of various moieties and their impact on accelerated wound healing[15,52,53]. The present investigation results are following the findings of the previous researchers, where L-glutamine exerts its beneficial effect via an inhibition in the STZ-induced depleted levels of SOD, GSH and Nrf2[25].

Diabetic wound associated with down-regulated hydroxyproline and Ang-1 production, which is in line with the previous evidence[54]. Administration of L-glutamine significantly augmented the hydroxyproline levels and Ang-1 mRNA expression suggesting its role in accelerated wound healing. Hydroxyproline is an important hallmark for collagen synthesis and degree of matrix formation, thus directly correlated with the rate of wound contraction. Fibroblast proliferation has a significant role in stimulating collagen formation and wound strengthening[18,55]. However, hyperglycemia significantly affects fibroblast proliferation and hydroxyproline synthesis, which further alter angiogenesis[18]. Along with hyperglycemia, elevated oxidative stress has been suggested as an important contributor to the defective angiogenesis process during DFU[56,57]. The angiopoietin (Ang-1) plays an essential role in the re-epithelialization, angiogenesis and development of new blood vessels known as neovascularization[58]. Thus, Ang-1 has a critical role in the growth and maintenance of vasculature during wound healing[59]. Elevated expression of Ang-1 has been suggested as a crucial pathway during accelerated wound healing[59]. Previously also Goswami *et al.*[30], reported the wound healing potential of L-glutamine via improved production of hydroxyproline and present study findings following findings of the previous investigators[30].

Clinically, L-glutamine has shown its potential against catabolic disorders, intestinal toxicity[21] and catabolic surgical patients[27]. Furthermore, its wound healing activity has been well documented in various experimental animal models of wounds[28-30]. Thus, L-glutamine can be considered a promising therapeutic moiety to promote wound healing in diabetic patients.

CONCLUSION

Metabolic abnormalities such as diabetes mellitus cause delayed wound healing. In the present study, administration of L-glutamine, an antioxidant moiety, demonstrates its wound healing potential against diabetic foot ulcers in experimental rats. L-glutamine accelerates the wound healing process acting via multiple mechanisms, including inhibiting elevated blood glucose levels, which suppresses the release of reactive oxygen species, thus inhibiting elevated oxidative stress. Furthermore, the Down-regulation of oxidative stress at wound area by L-glutamine decreases the release of inflammatory mediators, which further improves angiogenesis, thus improving wound healing during the diabetic condition.

SIGNIFICANCE STATEMENT

To the best of our knowledge, the findings of the present study first time reported putative mechanism of action of L-glutamine against diabetic foot ulcers in experimental rats. The present findings will deliver valuable information to researchers and physicians to find the alternative healthcare product to manage diabetic foot ulcers. Thus, these new findings on the possible role of L-glutamine may offer promise to better wound healing outcomes during diabetic foot ulcers.

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