RESEARCH ARTICLE

Clinical and histological study of cold physical plasma jet in treatment of full thickness skin wounds of normal and diabetic dogs

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Abstract

Objective: To study the effect of physical plasma jet in treating open wounds in diabetic and non-diabetic dogs.

Method: The experimental study was conducted at the College of Veterinary Medicine, University of Baghdad, Iraq, from 20 January, 2020 to 1st May 2020, according to (no. 1364/ P.G), and comprised adult male diabetic and non-diabetic dogs. They were divided into non-diabetic group N and diabetic group D. Each group was further divided into treatment subgroup T and control subgroup C. Each dog was subjected to 4 wounds 3×3cm in size. Home-made helium non-equilibrium atmospheric pressure plasma jet therapy was used for healing purposes. Clinical parameters were evaluated by observation, while histological images were scored based on the semi-quantitative evaluation of histological sections on days 3-, 7- and 21-days post-wound. Data was analysed using SPSS Version 26 with One-way ANOVA for statistical analysis between groups and sub-groups.

Results: Of the 24 dogs, 12(50%) were in each of the two groups, which were further divided into subgroups having 6(50%) dogs each. The therapy accelerated the process of wound healing in the NT and DT subgroups compared to NC and DC. Clinical observations revealed early and complete closure of plasma-treated wounds in NT and DT subgroups started at day 30 post-wounding, while in the NC subgroup it started at day 35, and in the DC subgroup, wounds failed to close even after 35 days post-wounding. Histological analysis suggested that a plasma jet supported epithelisation, angiogenesis, formation of new hair follicles and collagen fibres, while it also controlled inflammation. In addition, in NT and DT subgroups, it increased the proliferation of fibroblasts and deposition of collagen, and there is a very, highly significant difference between groups ($P<0.001$), and a significant difference between days ($P<0.05$).

Conclusions: Home-made helium non-equilibrium atmospheric pressure plasma jet therapy improved the quality and pace of wound healing.

Key Words: Helium, Hair Follicle, Wound Healing, Collagen, Diabetes, Inflammation, Fibroblasts, Proliferation, Angiogenesis

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Introduction

Severe skin injury can be life-threatening. Cutaneous wounds show an extraordinary mechanism of cellular function that is distinctive in nature. The repair process contains the interaction of cells, growth factors and cytokines[1]. Open wounds heal by the second intention, and this had been considered the treatment of choice for centuries[2]. The scar is the efficient newly-formed tissue, but it does not have the characteristics and functions of the physiological tissue that it replaces. Diabetes mellitus (DM) is one of the common endocrinopathies in dogs[3]. It aggravates wound healing. The high glucose environment leads to poor re-epithelialisation and angiogenesis by disrupting the interactions of growth factors, like insulin-like growth factors (IGF), and vascular endothelial growth factors (VEGF). Besides, chronic wound healing results from any delay during the four wound-healing phases. As such, shortening the chronic wound healing process is a critical issue in modern dermatology[4]. Also, cold atmospheric plasma (CAP) used in wound healing are multi-component and complex systems that contain unique features, while being dose-dependent. CAP is an important source of highly reactive oxygen species (ROS), as well as reactive nitrogen species (RNS). These reactive components play a beneficial role in the healing process. They can improve re-epithelialisation, granulation formation and angiogenesis [5]. Likewise, non-thermal plasma (NTP) can improve epithelialisation, stimulate reparative collagen synthesis and deposition, and enhance excisional wound closure, protecting the skin from aging by the production of nitric oxide (NO)[6].

Also, in the early stages of repair, CAP treatment modulates the inflammation stage, while in the later stages, it seems to avoid post-traumatic skin disorders during scar formation. After >12 months of follow-up, no

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precancerous skin features occurred in a study[7]. CAP is functional for mechanical strength improvement of repair tissue which correlated with wound closure, and rising of tissue tolerance against rupture which owes therapeutic value in clinical studies. In addition, CAP can cause a reduction of bacterial bioburden or open wound size, which are part of chronic wounds[8].

The current study was planned to study the effect of physical plasma jet in treating open wounds in diabetic and on-diabetic dogs.

Materials and Methods

The experimental study was conducted at the at the Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Baghdad, Iraq, from 20 January, 2020 to 1st May 2020 and comprised adult male dogs. After approval from the institutional ethics review committee, all procedures were conducted in line with the guidelines for the humane care of laboratory animals (no. 1364/ P.G).

The animals were divided into non-diabetic group N and diabetic group D. Each group was further divided into treatment subgroup T and control subgroup C. In group D, the dogs were subjected to experimental induction of diabetes. The dogs were kept on a fast for 24 hours before the induction of diabetes. Alloxan was injected intravenously (IV) after dissolving in normal saline 0.9% at a dose of 100mg/kg. After 32 hours, the dogs showed fasting hyperglycaemia.

The treatment subgroups were treated with home-made non-equilibrium atmospheric pressure plasma jets (N-APPJ). All surgical procedures were performed under aseptic conditions and general anaesthesia[9]. Each dog was subjected to 4 cutaneous full-thickness surgical wounds 3×3cm in size; 2 wounds on each side of the animal's back. The wound healing process was evaluated histopathologically, and wound biopsy was obtained on days 3, 7 and 21 post-wounding, and clinically by daily monitoring of the animals and the wounds for 30 days post-wounding.

N-APPJ, a device that generates plasma using helium gas, was used with a distal end of the Pyrex tube (DBD) configuration device (Figure 1). The distance was 15mm between DBD and the wound surface, the power density was 44mWatt/cm^2, and plasma radiation energy dose was 76J/cm$^{2.10}$. All wounds were indirectly exposed twice to the plasma jet at 0 and 1 post-wounding in line with literature[11].

Clinical observation for wounds was made in each phase on days 3, 7 and 21.

Biopsies of 1cm^3 were obtained from the sites of the wound bed and periphery. The slides were stained with haematoxylin-and-eosin (H&E) and Masson's trichrome stain. Histological images were scored based on semi-quantitative evaluation of the histological sections[12]. Data was analysed using Microsoft, Excel, Minitab, v17; IBM SPSS V26. Results mentioned in the present study as mean ± SE. Student Independent t-test to test the means between two groups, one, way, analysis of variance (ANOVA) were, used to find changes between groups or days after treatment[13].

Results

Of the 24 dogs, 12(50%) were in each of the two groups, which were further divided into subgroups having 6(50%) dogs each. In group N, NC animals showed signs of

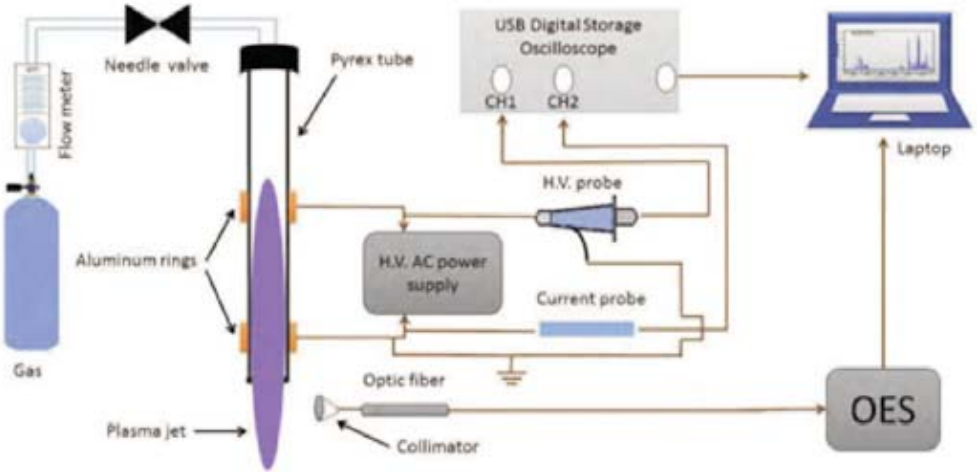

Figure-1: Schematic representation of the distal end of the Pyrex tube (DBD) plasma jet system.

Table: Scoring of the histological sections according to the semi-quantitative evaluation among the groups during wound healing stages.

Days	Histological Parameter	NC	NT	DC	DT	P-value¥
		Mean ± SD±SD	Mean ± SD±SD	Mean ± SD±SD	Mean ± SD±SD	
3 days	Inflammatory infiltration	1.50 ± 0.58±0.58	1.25 ± 0.50±0.50	2.75 ± 0.500.50	1.00 ± 0.001.00 ± 0.00±0.00	0.018*
	P-value	0.543			0.001***	
	Amount of granulation tissue.	2.75± 0.50±0.58	1.50 ± 0.58	3.50 ± 0.58±0.58	1.75 ± 0.500.50	0.001***
	P-value	0.002**			0.004***	
	Collagen fiber orientation	1.00 ± 0.00±0.00	1.00 ± 0.00±0.00	-	1.00 ± 0.00±0.00	1.00
	P-value	1.00			b**	
	Pattern of collagen	1.00 ± 0.00±0.00	1.75 ± 0.500.50	-±0.00	1.25 ± 0.50±0.50	0.022*
	P-value	0.024*			b**	
	Amount of early collagen	2.50 ± 0.58±0.00	1.25 ± 0.500.50	4.00 ± 0.0±0.50	1.25 ± 0.50±0.50	0.001***
	P-value	0.002**			0.001***	
	Amount of mature collagen	-	3.00 ± 0.000.0	-	3.00 ± 0.00±0.00	1.50
	P-value	a***			a***	
7 days	Inflammatory infiltration	1.75 ± 0.500.50	2.00 ± 0.00±0.00	2.25 ± 0.00±0.50	2.00 ±0.50±0.00	0.103
	P-value	0.356			0.356	
	Amount of granulation tissue.	1.50 ± 0.58±0.58	2.00 ± 0.00±0.00	2.75 ± 0.82±0.50	1.75 ± 0.58±0.50	0.012*
	P-value	0.134			0.030*	
	Collagen fiber orientation	1.50 ± 0.50±0.00	2.00 ± 0.00±0.00	1.00 ± 0.000.00	1.25 ±0.50±0.50	0.001**
	P-value	0.134			0.442	
	Pattern of collagen	1.50 ± 0.580.58	2.00 ± 0.00±0.00	1.00 ± 0.00±0.50	1.50 ± 0.58±0.58	0.073*
	P-value	0.134			0.135	
	Amount of early collagen	1.50 ± 0.50±0.50	2.00 ± 0.00	3.75 ± 0.50±0.82	2.00 ± 0.00±0.00	0.036*
	P-value	0.124			0.01**	
	Amount of mature collagen	3.00 ± 0.00±0.00	2.00 ± 0.0±0.00	-	2.75 ± 00	0.006**
	P-value	b**			a***	
21 days	Inflammatory infiltration	2.50 ± 0.50±0.50	3.00 ± 0.00±0.00	2.00 ± 0.00±0.00	2.70± 0.58±0.58	0.010**
	P-value	0.375		0.024*		
	Amount of granulation tissue.	2.75 ± 0.50±0.50	3.75 ± 0.50±0.50	2.00 ± 0.00±0.00	3.00 ± 0.00±0.00	0.001***
	P-value	0.002**			b**	
	Collagen fiber orientation	2.00 ± 0.50±0.50	2.75 ± 0.50±0.50	1.50 ± 0.58±0.58	2.50 ± 0.58±0.58	0.004**
	P-value	0.024*			0.030*	
	Pattern of collagen	2.25 ± 0.50±0.50	3.00 ± 0.00±0.00	1.75 ± 0.50±0.50	3.00 ± 0.00±0.00	0.001***
	P-value	0.024*			0.002**	
	Amount of early collagen	2.75 ± 0.50±0.50	3.75 ± 0.50±0.50	2.50 ± 0.00±0.00	3.25 ± 0.82±0.82	0.001***
	P-value	0.030*			0.05*	
	Amount of mature collagen	2.00 ± 5.00±0.0	1.25 ± 0.50±0.50	2.50 ± 0.82±0.82	1.50 ± 0.50±0.50	0.004**
	P-value	0.024*			0.032*	

restlessness reflected by rubbing their back with the ground, and its severity gradually decreased as the wounds continued to heal. The wounds appeared more blood-oozing than NT wounds. The blood coagulation effect of the jet of non-thermal plasma did not appear immediately, but it appeared gradually after 24 hours during preparation for the second dose. The wound bed of NT healed with regular shapes and thickness, and were smaller with less remarkable scar formation in comparison with NC. Complete closure in NT was seen at

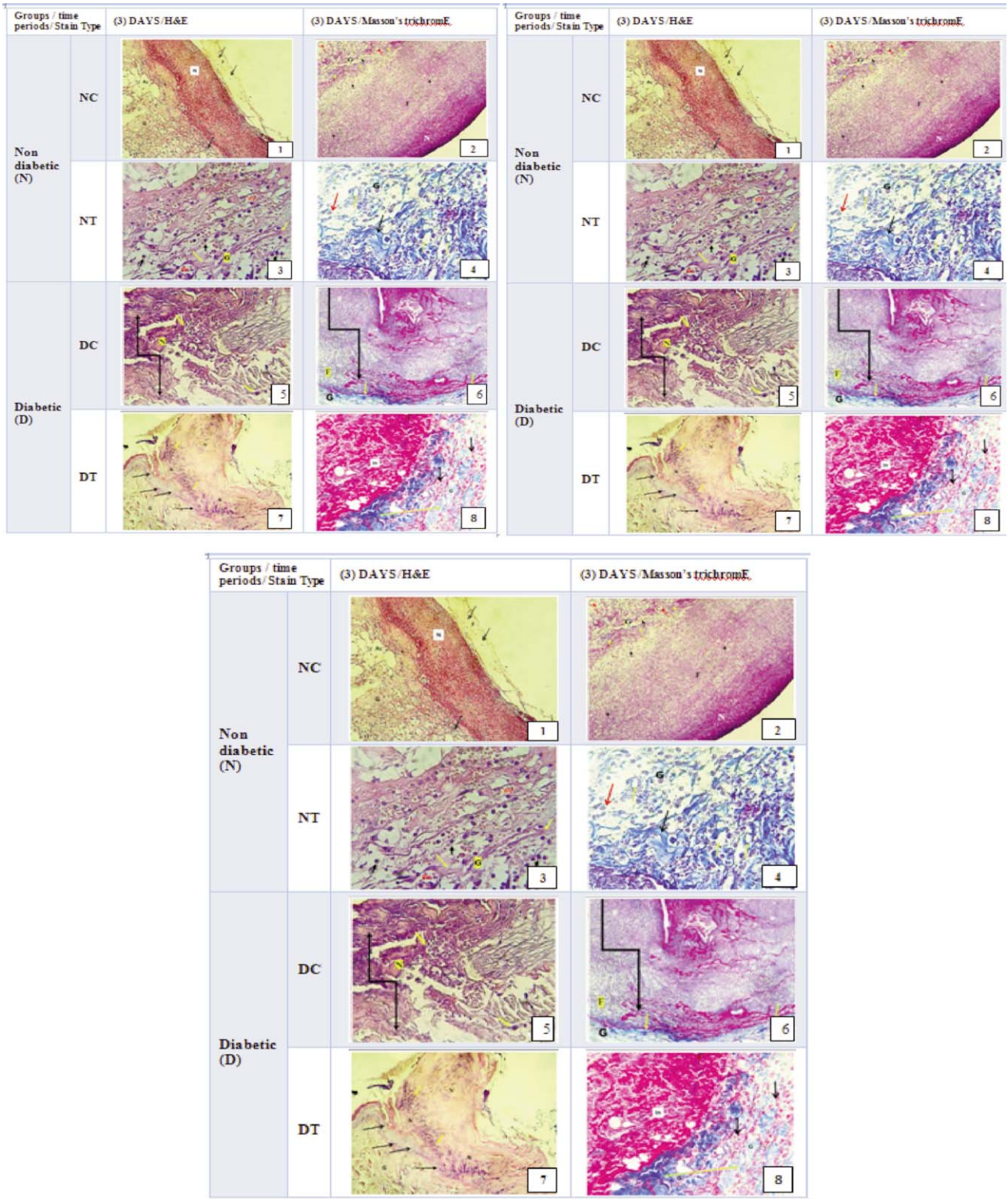

Figure-2: Histopathological sections in diabetic and non-diabetic groups. 1-A: At 3 days postoperation,(1): NC; fibrin networks (F) plenty of inflammatory cells (arrows), neovascularised granulation tissue (G), necrotic tissue debris (N), adipocytes (Ac) (10X). 2: NC; fibrin networks (F), plenty of neutrophils (black arrows), vascular granulation tissue (G), blood vessel (red arrow), moderate early collagen fibres (yellow arrows), under necrotic debris (N) (10X). 3: NT; full thickness wound characterised by plenty of inflammatory cells (black arrows), profound granulation tissue (G), reticular pattern of profound amount of early collagen fibres (yellow arrows), and new vascularisation filling the wound gab (BV) (40X). (4)

NT; immature granulation tissue (G) circle congested blood vessels (yellow arrow), containing inflammatory cells (red arrow), a profound amount of early collagen fibres light blue colour (black arrow) mixed orientation and reticular pattern (40X). (5): DC; few inflammatory cells (yellow arrows), fibrin networks (F) undernecrotic debris (N), no granulation tissue (40X). (6): DC: necrotic debris (black arrows), fibrin networks (F), granulation tissue (G), few collagen fibres (yellow arrow) (40X). (7): DT: thin tongue of epithelial cells (black arrows), inflammatory cells (violet arrows), necrotic tissue (N), moderate vascular granulation tissue (G) (10X). (8): DT; vascular (black arrows), granulation tissue (G), (yellow arrow) collagen fibre reticular pattern, inflammatory exudates (In) (40X). (1-B): At 7 days postoperation: (1): NC; profound vascular (Bv) granulation tissues (G), vertical orientation and reticular collagen fibre pattern (red arrows), moderate inflammatory cells (black arrows) (40X). (2): NC; neutrophils and mononuclear cells (black arrows), fibrin and immature granulation tissue (G) high vascularisation (yellow arrow), few mild to moderate blue stain collagen fibres (40X). (3): NT; 3-4 cells thickness epithelial layer (black arrows) overmoderate amount of granulation tissue (G), mixed, collagen fibres, orientation, and pattern (blue arrow), a moderate amount of inflammatory cells (red arrows), mononuclear cells (blue arrows) extended under necrotic tissue (N) (10X). (4): NT; granulation tissue and dense blue colour collagen fibres at the deep part of the wounds (yellow arrow), moderated colour collagen fibres in upper part (black arrow) (40X). (5): DC; inflammatory cells (neutrophils) (arrow), cellular debris (N), severe haemorrhage (H), (40X). (6): DC; less intensity blue colour of granulation tissue (G) started to replace mass of fibrin networks (yellow arrow) under necrotic cellular debris (N) (10X). (7): DT; 2-3 thickness epithelial layer (E) covered by keratine (K), rete-edge (black arrow), sebaceous glands (Sg), moderate granulation tissue (G), inflammatory cells (red arrow) (40X). (8): DT; vascular (bv) granulation tissue (G), profound earlycollagen fiber reticular pattern (black arrows), mild to moderate intense blue colour near the epithelial layer (red arrow), moderate intensity of these colours in deep part of the wound (yellow arrow) (10X). (1-C): At 21 days postoperation: (1): NC; complete epidermal layers (E) with rete-ridge under necrotic tissue (N) mature granulation tissue (G) (40X). (2): NC; mature granulation tissue expressed high-density blue colour horizontal orientation, reticular pattern collagen with minimal early collagen fibres, and moderate mature collagen fibres (arrows) (40X). (3): NT; complete epidermal layer (E) over scanty immature granulation tissue (G) few blood vessels, less cellular. Mature collagen fibre fascicles, with horizontal orientation (yellow arrows), hair follicle (H), sebaceous (SB), and sweat glands (S) (10X). (4): NT; fully-developed epidermal layer (E) rete-ridge (arrow) overmature granulation tissue of dermis (D) (40X). (5): DC; proliferation of epithelial cells 2-3 cells thickness (E), under the necrotic tissue (N) and overinflammatory reaction (arrow), vascular granulation tissue (G) (40X). (6): DC; tongue of epithelial cells (red arrow), overvascular granulation tissue (G) (10X). (7): DT; full thickness epidermal layer with rete-ridge (E) overmature granulation tissue, hair follicle (H) (10X). (8): DT; dark blue stained fascicle pattern collagen fibres (C), mature granulation tissue (G) and blood vessels (Bv) (40X).

30 days post-wounding, while NC wounds showed complete closure at 35 days post-wounding.

In group D, DT and DC dogs appeared less active, with an increase in the incidence of bleeding during surgery. DC dogs showed signs of restlessness, and the wounds appeared with fewer signs of inflammation at 3 days post-wounding. DT dogs appeared in less pain and had less blood-oozing, with increased signs of inflammation than DC dogs. DT wounds healed completely, and complete closure of wounds started at 30 days post-wounding.

Histopathological scoring and intergroup comparisons showed significant differences (Table; Figure 2). The difference in D &N groups and the controls and treatment sub-groups was also assessed in the newly formed skin type by assessing the number of inflammatory cells, granulation tissue, type of collagen fibres and its maturation. One-way ANOVA was used for statistical analysis between groups and sub-groups.

Discussion

Clinical observation suggested that NC animals had signs of restlessness for several days post-wounding, but this was not noted in NT animals. Recent studies have investigated the role of CAP in reducing negative effects and in accelerating the healing process[14]. The ability of CAP to control bleeding during the first 24 hours was detected in the current study, which matched earlier results[15]. A study[16] analysed bovine blood smear histologically, and revealed pro-coagulant effectiveness.

In the current study, the harmful effect of diabetes started with an inflammatory phase, and an increase in the incidence of haemorrhage was detected in diabetic wounds during and after surgery. A study reported diabetic complications, including changes in pro-coagulator microparticles, endothelial dysfunction, modified concentrations of coagulator proteins, hyper-activation of platelets, altered metal ion homeostasis, and changes in lipid metabolism[17]. Also, a recent study[18] highlighted positive effects of CAP in the primary inflammatory phase of wound healing.

In the current study, histopathological scoring showed the effect of CAP in the promotion of healing process. Studies have shown that CAP is a dose-dependent therapy for endothelial cell-mediated angiogenesis, and is used to increase proliferation of endothelial cells, fibroblasts and keratinocyte cells[19]. Arndt et al.[14] showed that the plasma treatment promoted acute inflammation that peaked at 4 days post-wounding, which was earlier than the control group. A study[20] mentioned that the NTP stimulates keratinocytes and fibroblasts, resulting in faster cell proliferation and migration, which can shorten the wound healing process.

In the current study, the regenerative effect of CAP was seen in NT at day 21 post-wounding, which was in line with an earlier study[8]. In the present study, NT dogs showed epithelialisation and angiogenesis more significantly than NC dogs on days 14 and 21. Also, after N-APPJ treatment, the inflammation was significantly less than that of the control wounds on day 21. However, a study[1] revealed that all the wounds of both treated and control groups were completely epithelialized at day 21, but the scar width in the plasma exposure group was smaller, with better re-epithelialisation that was evaluated histologically.

In the current study, DC wounds did not show signs of healing at 3 days post-wounding, while DT showed the initiation of the healing process. In literature, impaired repair of diabetic wounds has been associated with lower NO bioavailability[21]. A study[22] showed histo-morphological differences between treated and control groups, especially related to the epidermal layer of treated diabetic wounds which was not found in control wounds.

The effect of hyperglycaemia in delaying wound healing was obvious in the current study. Similar findings were reported by a recent study[23].

In the current study, DC wounds failed in terms of epithelial regeneration and even keratinisation. Similar results were reported earlier[24] In line with the current results, a study[25] reported that cold physical plasma with helium gas treatment accelerated the rate of wound healing in a diabetic murine model.

Conclusion

The clinical results corresponded with the histological findings, and together showed a good safety and effectiveness profile of the non-thermal atmospheric pressure helium plasma jet in the treatment of acute open wounds in non-diabetic and diabetic dogs.

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Conflict of Interest: None.

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