Comparative Study of Cold Physical Plasma Effect on Modulation of Basic-Fibroblast Growth Factor and Tumor Necrosis Factor Alpha in Full Thickness Skin Wound Healing Process in Normal and Diabetic Dogs

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Abstract

Non-thermal atmospheric pressure plasma (N-APP) a physical method that recently had been extensively studied by researchers as a possible therapy in biomedical researches such as wound healing. In clinical dermatology, cold plasmas are mainly used for the treatment of chronic wounds and pathogen-based skin diseases, in which stimulation of tissue repair and decontamination. In this research, home-made Helium Non-Equilibrium atmospheric pressure plasma jet (He -NAPPJ), that had been generated using a DBD configuration for exceptional standardization protocol of this plasma source that was used in treatment of full-thickness skin tissue wound of normal and diabetic dogs. This clinical research in veterinary medicine is the first one in Iraq that study role of cold physical plasma in wound healing in diabetic wounds used the dogs as a model. The results were evaluated by quantitative real-time polymerase chain reaction qRT-PCR, which showed enhancement in would healing process by cold plasma jet, by modulation of gene expression of basic-Fibroblast Growth Factor and Tumor Necrosis Factor alpha(TNF-α) in normal and diabetic dogs.

Key words: *Growth factor, Skin Wound healing, non-thermal plasma jet.*

Introduction

Plasma medicine is an emerging field incorporating physics, chemistry, life science, and medicine. To estimate the role of non-thermal plasma in wound healing process in this study, basic fibroblast growth factor (b-FGF) was used as a parameter, it plays a vital role in the repair of injured tissue in many studies,b-FGF has ability to promote cell division and proliferation related to injury repair and tissue reconstruction, moreover, during acute wound there is a rise in the production of b-FGF normally, which is responsible for angiogenesis, granulation tissue formation, re-epithelialization and tissue remodeling. Furthermore, Injury-induced b-FGF promotes the aggregation of monocytes, neutrophils, macrophages and fibroblasts via chemo taxis in the injured tissues (1). Tumor necrosis factor alpha (TNF-α) is a multifunctional pro-inflammatory cytokine, which is secreted mainly by monocytes and macrophages, the main supply of TNF-α are macrophages and T-cells, yet many other cells such as B-cells, neutrophils, and endothelial cells have been described to produce TNF-α. The functional relevance is broad, like the mediation of cell survival and pro-inflammatory response and, TNF-α instigates signaling pathways of the cell death (2).

Diabetes mellitus (DM) is one of the common endocrinopathy of dog characterized by hyperglycemia, glycosuria and weight loss. The treatments for diabetic wounds include surgical and non-surgical procedures. As a non-surgical treatment, the application of non-thermal plasma jet, is one of the promising methods to promote wound healing or to provide healthy wound beds for surgical treatments, a non-equilibrium

atmospheric pressure plasma jet (N-APPJ) is considered as environment friendly substances, have been studied all over the world, as reliable effects, safe, non-toxic and no adverse reactions have been reported in the treatment of wounds, but remains as dose dependent radiation. However, the interrelationship between diabetes, non-thermal plasma treatment and the wound healing process acts together with the immune system and the immunomodulatory function [3].

The aim object of this study is to demonstrate the effect of non-thermal plasma jet in treatment of diabetic wounds, through demonstration of tissue levels of gene expression of growth factors and cytokine using quantitative Real Time Polymerase chain reaction (qRT-PCR) technique, to measure the b-FGF and TNF-α gene expression level in alloxan- induced diabetic dogs compared to the normal ones. Also, studying the modulation effect of NAPPJ for both b-FGF and TNF-α.

Materials and Methods

Experimental design:

The experiments performed at the Department of surgery& obstetrics in the college of veterinary medicine, University of Baghdad, and all procedures conducted within the guidelines for humane care of laboratory animals and approved by the University of Baghdad ethics Committee (no. 1364/ P.G).

Twelve adult male dogs were used. All surgical and non-surgical procedures were performed under aseptic conditions and general anesthesia. Each dog was subjected to (4) full-thickness open cutaneous wounds of (3×3cm) of the animals back. Dogs were put into two groups: 1- The non-diabetic animal groups(**N**): (six) The dogs were divided in two sub-groups: Non diabetic control (NC);Non diabetic treated (NT) by(N-APPJ); 2- The diabetic animal groups (**D**): (six) dogs were subjected to experimental induction diabetes, and after two weeks from induction they underwent the same previous procedure (grouping, wounding and treatment), and the sub- groups were: Diabetic control (DC); Diabetic treated (DT) with (N-APPJ). All the wound healing process was evaluated by Tissue level detection of (b-FGF), and (TNF-α), in consequence periods (0, 3, 7, and 14 days) post-surgery.

Induction of Experimental Diabetes:

Six dogs were placed on a fasting regimen for 24 hours before the induction of diabetes via intravenous injection of Alloxan which was dissolved in normal saline 0.9 %, at a dose of 100 mg/kg. After 32 hours, the dogs showed fasting hyperglycemia.

Non-thermal Plasma Jet System (Experimental Setup):

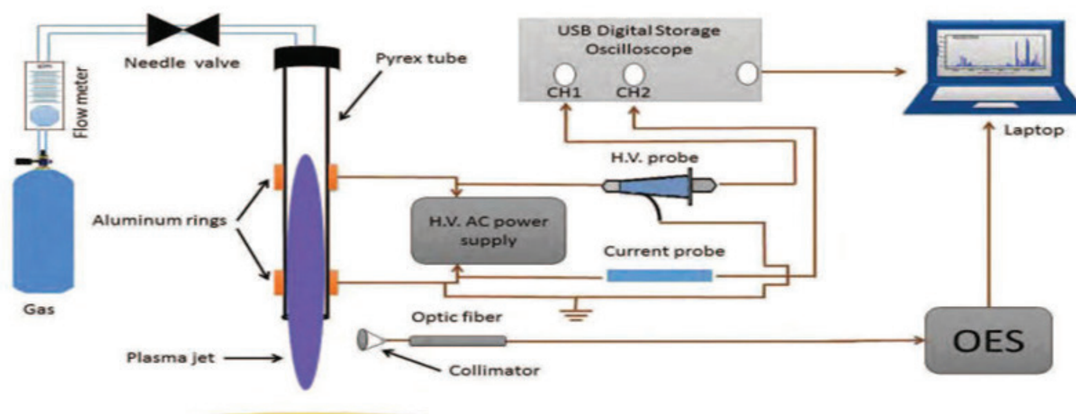

Figure (2.1): Schematic representation of the DBD plasma jet system.

In this research, the characterization of home-made Helium Non-Equilibrium atmospheric pressure plasma jet (He-NAPPJ), that generated using a dielectric barrier discharge (DBD) configuration device (figure 2.1), driven by a (8 kVp−p) voltage, the frequency (12 kHz). As a working gas, Helium (He) was used in flow rates (6) slm, with 32 °C He gas temperature, plume length 40 mm.The distance is 15 mm from the distal end of Pyrex tube (DBD) and the wound surface, the power density 44 mWatt/cm^2, and plasma radiation energy dose 76J/cm^2.

Molecular diagnosis:

The biopsies from skin tissue of each animal were collected from normal skin and wounds of all sub-groups, and kept at deep freezing (-80 °C), then evaluated using RNA extraction kit for extract m-RNA then Real Time Polymerase chain reaction (PCR) technique for house-keeping, (b-FGF) and (TNF-α) primers as mentioned below:

Primers: Three primers were used in this study including the specific gene of cutaneous tissue in dogs which including Anterior Reverse glyceraldehydes-3-phosphate dehydrogenase *(AR-GAPDH)* gene primer that was used as Housekeeping gene (HKG)[4], anterior reverse b-Fibroblast Growth Factor (*AR-FGF*) [5] and anterior Reverse (*AR-TNF-*α*)*genes primers [4] that were used as target genes. The primers were used in quantification of genes expression by using Real–Time quantification Polymerase Chain Reaction (q-RTPCR) techniques based BRYT Green DNA binding dye (Promega–USA).

The Kits used in molecular techniques as SaMag Total RNA Extraction Kits,theGoScript™ Reverse Transcription System kit (Como–Italy), The GoScript™ Reverse Transcription System kit (Promega- USA), and GoTaq® qPCR Master Mix kit (Promega-USA).

B. m-RNA Extraction: m-RNA was extracted by automated nucleic acid extraction system according to with SaMag Total RNA Extraction Kit by Sacace Biotechnologies Srl Como – Italy company instructions. The extracted m-RNA samples were kept at freezing temperature (-20) C°.

C. Examination of m-RNA Concentration and purity: The concentration of m-RNA was measured by using NanoDrop ND-1000 spectrophotometer (thermo-fisher, USA), and according to the manufacture ́s manual (NanoDrop Technologies, 2006).

D. cDNA synthesis for easy transition to gene-specific target amplified:

By using GoScript™ Reverse Transcription System KIT, and according to the manufacture ́s manual. The following procedure is designed to convert up to 5µg of mRNA Mix and briefly centrifuge each component before use: Experimental RNA (3µl/reaction); Primer Oligo (dT)15 (1µl/reaction); Nuclease-Free Water(1µl/reaction) the Final volume was (5µl) For each reaction. The PCR component for syntheses of cDNA: Nuclease-Free Water (to a final volume of 15µl)(7.3µl); GoScript™ 5X Reaction Buffer (4.0µl); MgCl2 (1.2µl); PCR Nucleotide Mix(1µl); Recombinant RNasin® Ribonuclease Inhibitor(5.0µl); GoScript™ Reverse Transcriptase(1µl) and the final volium was (15.0µl) amount for each reaction. 15µl aliquots of the reverse transcription reaction mix to each reaction tube were added to ice. a final reaction volume of 20µl per tube

Quantitative Real-Time PCR (q RT-PCR) master mix preparation:

Assembling the Reaction Mix: Prepared the reaction mix by combining the GoTaq® qPCR Master Mix with cDNA, PCR primers and Nuclease-Free Water, the component and their volume used in reaction mixed of qPCR, the target genes primers TNF-α, or b-FGF were used, also the calibrator gene HKG. Each q-RT-PCR mixture reactions were prepared with (10µl) of GoTaq® qPCR Master Mix, (1µl) Forward Primer (20X), (1µl) Reverse Primer (20X), (3µl) Nuclease-Free Water, (**5** µl) cDNA, and the final volume was (20µl). PCR program was used with Rotor-Gene cycler: the first step was (1) cycle of GoTaq ® Hot Start Polymerase activation at (95°C) for (2 minutes), then denaturation

was used (40) cycles at (95°C) for 15 seconds, finally, Annealing and extension programed at (60°C) for (1 minute).

Statistical Analysis: The data were analyzed using the following software, Microsoft excel, Minitab v17, and IBM SPSS V26. The results reported in this study were expressed as mean +_ SE. Two-way ANOVA were used to test between groups and days after treatments. Mean were compared using the least significant difference test (L.S.D).

The Results

Tissue level detection qRT-PCR:

The dogs in diabetic group were kept in hyperglycemic state. The results showed slight non-significant increase in expression of (b-FGF) in wound tissue by qPCR after 14 days from alloxan- induced diabetes. While at the same days the results showed significant increase in TNF-α level in wound tissue in diabetic dogs.

Fibroblast growth factor (b-FGF)& (TNF-α) detection:

Table (3-1): The final result is presented as the fold change of target gene expression a. (b_FGF), b. (TNF-α) in treatment groups by using $2^{-\Delta\Delta CT}$ method(6).

a b_FGF	Days after treatment				Mean groups¥	P-value
	0 days	3 days	7 days	14 days		
Non-Diabetic treatment	1.26±0.011	8.42±0.133	7.20±0.173	2.74±0.427	4.91±0.91	0.001***
Diabetic treatment	1.06±0.034	5.48±0.369	5.06±0.029	1.06±0.067	3.16±0.642	
Mean days¥¥	1.16±0.048	6.95±0.680	6.13±0.485	1.90±0.423		
P-value	0.001***					
LSD	Group=0.324		Days=0.458		Group*Days=0.648	

b TNF-α	Days after treatment				Mean groups¥	P-value
	0 days	3 days	7 days	14 days		
Non-Diabetic treatment	1.04 ± 0.017	3.02 ± 0.075	1.18 ± 0.104	1.06 ± 0.017	1.57±0.254	0.001***
Diabetic treatment	1.06±0.040	2.00±0.162	0.80±0.133	0.61±0.098	1.12±0.169	
Mean days¥¥	1.05±0.020	2.51±0.242	0.99±0.114	0.84±0.110		
P-value	0.001***					
LSD	Group=0.142		Days=0.201		Group*Days=0.285	

Data presented as mean ±SE, Two way a nova were used to test between groups and days, *, **, *** significant (P<0.05), highly significant (P<0.01), very high significant (P< 0.001) respectively.

Discussion

The results showed slight non-significant increase in expression of (b-FGF) in wound tissue by qPCR in hyperglycemic animals, while a significant increase in TNF-α level in wound tissue sample in diabetic dogs. This finding was explained by many researchers, who stated that the uncontrolled diabetes is characterized by hyperglycemia that has been shown to drive oxidative and nitrative stress. Hyperglycemia also stimulates expression of multiple survival factors, including (FGF-2), and vascular endothelial growth factor (VEGF),which is generally protective against endothelial cell death, which was similarly elevated in high glucose conditions [(7)]. **The elevation of in the** TNF-α level has been measured in diabetic laboratory animals (rats), in study [(8)] used streptozotocin-induced diabetic rats, with significant increase in TNF-α messenger RNA expression were twice as high in diabetic rats than in no diabetic control rats.

In the present study it has been found that there is a relationship between the results of both (b-FGF and TNF-α). In other study the researchers examined the possible role of basic fibroblast growth factor (b-FGF or PGE2) in regulating the effects of TNF-α, they find that FGF enhanced the amount of prostaglandin (PGE2) produced in response to TNF-α between (3-11) fold, and increases TNF-α receptor expression in fibroblast cells. Glucose and (TNF-α) concentrations are increased in diabetes, these factors correlated both with diabetic endothelial cell apoptosis, vasculopathy and induced cell viability inhibition that led to the proliferative inhibition. The b-FGF associated elevation in TNF-α-induced cell death. b-FGF release by endothelial cell in high glucose goes to cell cycle progression, which makes cells more liable to TNF-α-induced cell death [(9)].

In the present study the level of b-FGF gene expression appeared high in both treatment sub-groups (NT &DT) when compared to control sub-groups (NC& DC) significantly at ($P< 0.001$) between the periods of time 0, 3, 7, and14 days post wounding, also there was significant difference between groups (N&D) at ($P< 0.001$). In more details, the results showed high up-regulation of b-FGF gene expression (7.16) at 3 days post wounding compared with the zero day; (5.94) at 7 days, then normal regulation (1.48) at 14 days, post wounding in comparison with the zero day, in NT group attributed to NC group. Also in DT group attributed to DC group there were up-regulation of gene expression at (4.42) at 3 days and (4) at 7 days, while normal regulation in gene expression found at 14 days post wounding compared with the zero day. In a study [(10)] found that the endothelial cells treated with plasma for 30 seconds demonstrated twice as much proliferation as untreated cells, in that way they suggested that low dose non-thermal plasma enhances endothelial cell proliferation due to reactive oxygen species mediated b-FGF release. The same results found in [(11)] they suggested increase in b-FGF mRNA expression in murine skin wound tissue treated with CAP that was compared to control group**.** Also, the secretion of angiogenesis-related molecules is affected after the CAP treatment in endothelial cells, fibroblasts and keratinocytes.

This clinical study had proved the effect of AP-He-PJ in boost the gene expression of TNF-α in both treatment sub-groups (NT &DT) at 3 days, when compared to control sub-groups (NC& DC) significantly at ($P< 0.001$), also there was significant difference between groups (N&D) at ($P< 0.001$). There is significant difference in (TNF-α) gene expression of treatment sub-group NT in comparison to control NC between all periods of time, the same results found in DT in comparison to control DC between the periods of time. In the same way the results in NT sub-group showed up-regulation of TNF-α gene expression (1.98) at 3 days, while there was normal regulation in gene expression at 7 and 14 days post wounding compared with the zero day. However, in DT sub-group, there were up-regulation of gene expression at 3 days (6.42 ± 0.360), while there were slight down-regulation in gene expression at 7 and 14 days post wounding, when compared with the zero day. Many molecular researchers studied the TNF-α, they differ in explaining the role of this cytokine that involved in the process of wound healing. At wound tissues,

TNF-α is quickly released and initiates inflammation, synthesis of TNF-α was detected just after wound was developed, then increased during the first several hours, reached a peak level at day 1, and then reduced to the basal level, and they indicated that TNF-α is involved in the early healing process [12]. While other studies reported the harmful effects of TNF-α were significantly amplified in the presence of high glucose. The excessive TNF-α is closely related to diabetic complications by inducing endothelial cell apoptosis under high-glucose condition, which exacerbated inhibitory effects of TNF-α by increasing its expression [9]. A study [13] revealed the selective apoptotic effect of CAP on cytotoxic and T-helper cells, B-lymphocytes, and natural killer (NKT) cells may be involved in regulating the healing process, also, in vitro study by [11] demonstrated the beneficial effect of short time (60 s) application of NTP on parameters of molecules and cell function involved in wound healing in osteoblast-like cells. Their results showed the significant increase in the mRNA expression of pro-inflammatory cytokines IL-1β, IL-6, chemotactic factor IL-8, also, TNF-α and COX2 after 1 day compared to untreated cells. These mediators play an important role in wound healing managing the primary inflammation process, and the extra cellular matrix organization, which are both crucial for the entire wound regulation.

Conclusion

In conclusion, these findings not only support the fact that non-thermal plasma has a potent multifunctional agents during tissue regeneration, but also highlight the potential multimodulatory therapy advantages of non-thermal plasma. The enhancement in would healing process by cold physical plasma jet, by modulation of gene expression of basic-Fibroblast Growth Factor and Tumor Necrosis Factor-alpha in normal and diabetic dogs.

Conflict of Interest: None

Funding: Self

Ethical Clearance: Not required

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