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Histopathological study of using Fetal caprine acellular dermal matrix alone and in combination with Non-thermal plasma in healing of full-thickness acute skin wounds in bucks

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***Abstract*---**This study aimed to investigate the effect of non-thermal plasma (NTP) with Fetal caprine acellular dermal matrix (FCADM) scaffold in healing of the full-thickness acute skin wound in bucks. eighteen apparently healthy adult local breed bucks aged 1-2 years and weighing 25-30 kg were used. Each animal was subjected to creation of 4 full-thickness acute skin wounds on the shoulder region, two wounds on each side 10cm apart, the animals were divided into 3 groups, at first control group wounds were treated in routine way (cleaning the wound with normal saline every other day and dressing). The second group the wounds were treated with Fetal caprine acellular dermal matrix. The third group skin wounds were exposed to non-thermal plasma treatment with dose 6.6W/cm² for 30 sec (used probe 1cm in dimeter), then application of Fetal caprine acellular dermal matrix. The wounds were histopathologically evaluated at 3,7,21, 35 days post-operation using Hematoxylin & Eosin (H& E) and mason's trichrome stain. The results showed that the wounds treated with non-thermal plasma (group II) and scaffold (group III) healed faster wound healing compared with the control group (group I) without any treatment was significantly ($p < 0.05$). and the histopathological score was 14.16 in control group while in second group was 17.66 and in third one was 20. In summary, non-thermal plasma treatment had improved the healing efficacy of acute skin wounds without noticeable side effects. Non-thermal plasma can

support wound healing by its antiseptic effects, its stimulation of proliferation and migration of wound relating skin cells, and its pro-angiogenic effect. The Fetal caprine acellular dermal matrix scaffolds are currently believed to be a promising strategy for wound healing improvement. The collagen contained in Fetal caprine acellular dermal matrix is aware of playing a pivotal role in wound healing, and it is capable of inducing cellular regeneration. Moreover, it could promote the reconstruction. Therefore, it could facilitate dermis reconstruction, shorten epithelialization time.

***Keywords*---**histopathological, fetal caprine acellular, dermal matrix, Non-thermal plasma, full-thickness acute.

Introduction

The full thickness injuries are characterized by the complete destruction of epithelial regenerative tissues and due to lack of epithelialization leads to extensive scarring (Herndon et al. 1989) . The large wounds that cannot be corrected by conventional surgical procedures requires substitutes of missing tissue to keep the wound free of infection, to reduce pain and ensure early wound healing (Ruszczak 2003). Plasma is defined as a partially or completely ionized medium that is composed of many elements such as charged particles (electrons, ions), reactive oxygen species (ROS, e.g., ozone – O3), reactive nitrogen species (RNS), and UV radiation (Emmert et al. 2013; Fridman et al. 2008; Fridman et al. 2005). In recent years the application of non-thermal plasma in biomedical researches such as wound healing, blood coagulation (Tian and Kushner 2014), angiogenesis suppression (Gweon et al. 2013), cancer treatment (Panngom et al. 2014) and deactivation of microorganisms (Matthes et al. 2013; Ehlbeck et al. 2010) like MDR bacteria (Daeschlein et al. 2015) and even viruses (Ahlfeld et al. 2015) is rapidly growing, primarily due to its bactericidal properties. A large volume of studies has identified the positive effects of non-thermal plasma on healing acne (Chutsirimongkol et al. 2014) and eczema (Tiede et al. 2014), burn wounds (Lee et al. 2016), venous ulcers (Emmert et al. 2012), chronic leg ulcers (Ulrich et al. 2015), full-thickness cutaneous wounds in healthy and diabetic mice (Jacofsky et al. 2014), and skin rejuvenation (Brun et al. 2014). Unfortunately, the main mechanism of the non-thermal plasma interaction with cells or living tissue still remains unknown (Kalghatgi et al. 2011), but the effects of non-thermal plasma on the key wound-related cells have been reported in several studies. These studies have shown that plasma induces fibroblast migration and proliferation (Kalghatgi et al. 2010), promotes the proliferation of endothelial (Arndt et al. 2018) and keratinocyte (Haertel et al. 2014) cells, as well as the growth of epithelial cells (Hoentsch et al. 2011); these effects are attributed to the production of intracellular reactive oxygen species (ROS) (Dunnill et al. 2015). In addition, NO-containing plasma induces skin acidification (pH reduction) as a result of NOx interaction with water and increases dermal microcirculation (Heuer et al. 2015). These effects are similar to those found in the natural process of wound healing. Therefore, plasma therapy represents a promising new medication against chronic/infected wounds.

The scaffolds include synthetic biodegradable polymers, natural polymers and natural matrices derived from decellularized tissue (Uygun et al. 2010). The natural bio-scaffold have advantages over synthetic material that its mimic natural extracellular matrix (ECM) structure and composition, simulate natural stimulatory effects of ECM on cells and allows the incorporation of growth factors and other matrix proteins to further enhance cell functions. The scaffold allows cell invasion, their proliferation and secretion of own extra cellular matrix for longer duration leading to a complete and natural tissue replacement (Smith et al. 2000). Dermal substitute scaffolds promote fibroblast adhesion, growth and infiltration which accelerate and enhance dermal and epidermal regeneration (Lamme et al. 2000). The porosity, pore size and pore structure of the scaffolds are important for nutrient supply and more infiltration of fibroblast cells (Whang et al. 1995). Decellularized mammalian tissues that retain extracellular matrix components (i.e., collagens) have recently been reported to be an effective clinical strategy to treat difficult-to-heal wounds, The scaffolds helped the adhesion, proliferation, migration and differentiation of the cells, all of which were deemed as the extremely desired features for tissue engineering. The scaffold allows cell invasion, their proliferation and secretion of own extra cellular matrix for longer duration leading to a complete and natural tissue replacement. Dermal substitute scaffolds promote fibroblast adhesion, growth and infiltration which accelerate and enhance dermal and epidermal regeneration. The ECM mainly consist of proteins, including collagen, fibrin, fibrinogen, gelatin, elastin, etc., and polysaccharides, especially alginates, hyaluronic acid, cellulose, chitosan, etc., this complex mixture offers mechanical and biochemical support to surrounding cells and controls their performance in regeneration (AL-Bayati et al. 2016; Al-Falahi et al. 2018; Cornwell et al. 2009).

The authors chose to use an acellular fetal dermal repair scaffold to treat difficult-to-heal wounds based on its unique composition of type I and type III collagen found only in fetal tissue. During fetal development, and in healing cutaneous wounds, relatively high levels of type III collagen are present. In fetal dermis, type III collagen comprises approximately 30% of the total collagen, whereas in adult skin, it comprises 10% or less (Smith et al. 1986). Notably, only fetal tissues having such a unique collagen composition have been found capable of healing without scar tissue formation (Ramshaw 1986). The predominance of type III collagen in the early phases of adult cutaneous wound healing has long been appreciated (Larson et al. 2010). In the proliferative phase, type III collagen increases to 30% of the total collagen content. Wound healing experiments with adult mice genetically deficient in type III collagen demonstrated that diminished or low levels of type III collagen during wound healing results in increased scar formation and decreased tissue regeneration (Witte and Barbul 1997). Type III collagen, or, more specifically, the ratio of type III to other collagens, appears to be a key mediator and contributor to skin tissue development, healing, and regeneration. Published case studies and case series illustrate the clinical utility of the fetal dermal collagen scaffold technology.

The scaffold was reported to successfully augment secondary closure of a full-thickness skin injury with exposed bone and tendons following a crush injury (Ehrlich and Krummel 1996a). In another case presentation, sheets of the scaffold were stacked to fill significant tissue voids. The implanted dermal scaffolds

provided tissue bulk, and eventually supported a delayed skin graft in the effective treatment of a significant tissue deficit following aggressive debridement in a necrotizing fasciitis case (Ehrlich and Krummel 1996b). A case series on deep dermal wounds reported faster healing with reportedly less scarring when compared to treatment with negative pressure wound therapy (Reinke and Sorg 2012). To date, the largest case review published on fetal dermal collagen treatment compared outcomes of diabetic foot and venous leg ulcers treated with either fetal dermal collagen or a living skin substitute. The fetal dermal collagen was found to heal both ulcer types approximately twice as fast as the living skin substitute. The fetal bovine dermal repair scaffold, which is derived from fetal bovine dermis, was adopted by the authors' practice with the goal of healing wounds that had failed to heal using conventional treatments. When primary wound closure was not an option, and skin defects failed to re-epithelialize, aggressive surgical debridement was performed and the dermal repair scaffold was fixed into the wound bed. The product was observed to incorporate into the wound as re-epithelialization progressed (Volk et al. 2011).

Materials and Methods

Experimental Animals

In the current study, eighteen apparently healthy adult local bucks aged 1-2 years and weighing 25-30 kg were used. Two weeks before starting of the experiment, all animals were housed in pens of farm of animals at the College of Veterinary Medicine/ University of Baghdad. The animals were classified and numbered according to the experimental design, each animal was dewormed with albendazole at dose 10 mg/kg., body weight to avoid the enterotoxaemia, they were injected subcutaneously with a dose of 5ml of Co-Baghdad vaccine.

Experimental design

Each animal of 18 was subjected to creation of 4 wounds, on the shoulder region, two wounds on each side 10cm apart, the animals were divided into 3 groups:

- First group (control 6 animals/24 wounds): wounds were treated in routine way (cleaning the wound with normal saline every other day and dressing) Figure 4.
- Second group (FCADM 6 animals/24 wounds): the wounds were treated with caprine fetal dermal acellular matrix fixed at the 4 corners of the wound.
- Third group (NTP &FCADM 6 animals/24 wounds): the wounds were treated with NTP with dose 6.6W/cm² for 30 sec (using a probe 1cm in dimeter) (Jesus et al. 2016; Chanchai et al. 2014), then application of CFADM.

Fetal caprine acellular dermal matrix preparation

The fetus was obtained from pregnant goats for a full period and after cesarean section, the fetus was taken to prepare the cellular skin matrix, where the skin of the fetus was exuded. The skin was rinse gently with saline solution to get rid of sticky blood. Mechanical cleaning was completed manually to get rid of all

unwanted fats and connective tissues using dry gauze and keeping the fetus skin in freezing at -20°C. According to method described by (Aaron et al. 2016), frozen skins were thawed, and the epidermal side was gently scrape with a scalpel to remove the stratum corneum of skins and placed in 0.25% Trypsin-EDTA at 37°C for 90 min. All subsequent steps were performed at room temperature on an orbital shaker. Skin was washed in ddH2O thrice for 15 min and then incubated in 0.1 M NaOH (Macron) for 16 hrs. The skin was washed in Dulbecco's phosphate-buffered saline until the pH of the rinsing solution was neutral. If the skins received an extended wash, they were washed in serum-free DMEM with 1% penicillin and streptomycin on a rotating shaker at 37°C for 24 h (Figure 1).

Figure 1. preparation of the Fetal caprine acellular dermal matrix a. fetus skin after skinning b. shaker at 37°C c. final form

Surgical procedure

All the animals were medicated with penicillin-streptomycin in a dose of 10.000 IU and 10mg/kg B.W., respectively, withholding of food for 24 hours and water for 12 hours. The shoulder region was prepared surgically. Animals were sedated with xylazine: 0.1mg/kg IM and local infiltration anesthesia 1ml/1cm, 2 wounds (3*3cm) full cutaneous open wound were created on each side Wounds of the 1st group treated by cleaning the wound every other day with warm sterile normal saline followed by dressing of wound, while in the 2nd group the wounds were treated by application of CFDAM by fixing each scaffold at the corners of the wound using 3.0 monofilament nylon suture material. In the last 3rd group, the wounds were exposed to NTP at the previously mentioned dose then the bio-scaffold was applied as in the 2nd group.

Evaluations

Histopathological evaluation: The histopathological examination was done at 3,7,21, 35 days post-operation using H&E and mason's trichrome stain. All tissue samples were fixed with 10% buffered formalin, processed, embedded in a paraffin as the supporting medium. The tissue block was sectioned at 5-mm thickness and tissue section was placed on a microscope glass slide for histological staining with H&E, Masson's trichrome.

Equipment

Non- Thermal Plasma system which is fed by continuous voltage equal to 12V, the output voltage is 25KV and its frequency 30kHz, and 0.5mA (Figure 2), this device was manufactured by Department of Physics College of Sciences for Girls, University of Baghdad. (The patent is 5688).

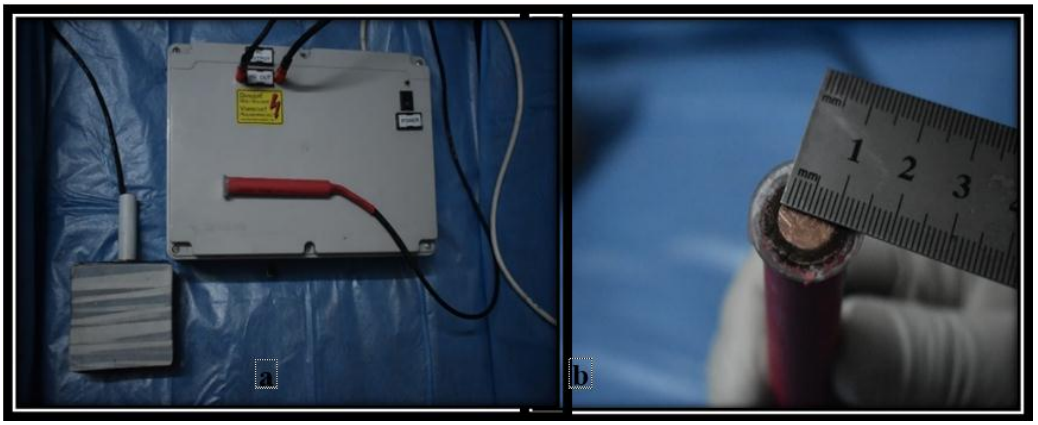
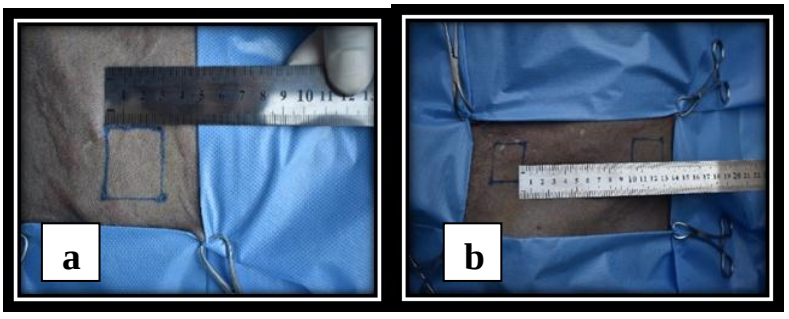


Figure 2. a. Photo of a portable NTP device b. NTP device probe 1cm diameter

Treatment of Wound

After inducing the wounds, the 1st group were treated by cleaning the wound every other day with warm sterile normal saline then dressing them, while in the 2nd group the wounds were treated by application of CFDAM by fixing each scaffold at the corners of the wound using 3.0 monofilament nylon suture material (Figure 3). In the last 3rd group the wound were exposed to NTP at the previously mentioned dose then the bio-scaffold was ap plied as in the 2nd group. Post to the NTP and CFDAM treatment, PBS was administered topically to the wounds. The same was done on the control lesions. After the treatment, the lesions were bandaged with sterile paraffin gauze using the "wet-to-dry" method. This method provides adequate wound protection and coverage, helps maintain a moist wound environment, and absorbs moderate amounts of wound exudates without drying up which could result in unnecessary pain during the next treatment and change of bandages. The bandaging is shown in Figure 4.



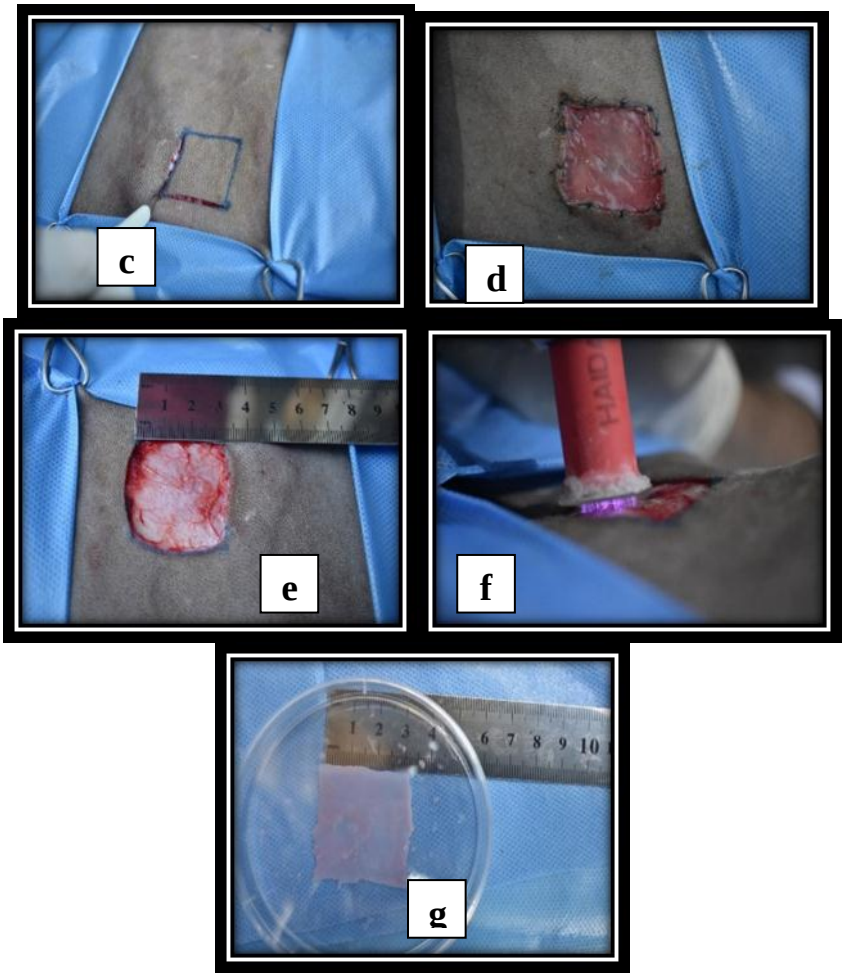


Figure 3. shows steps of wound creation and treatment, a. measurement of wound (3*3cm), b. distance between wounds (10cm), c. creation full cutaneous open wound, d. measurement of wound after creation, e. application of NTP at 0. 3cm from wound bed, f. CFDAM as same wound measure, g. CFDAM after fixation at the wound corners

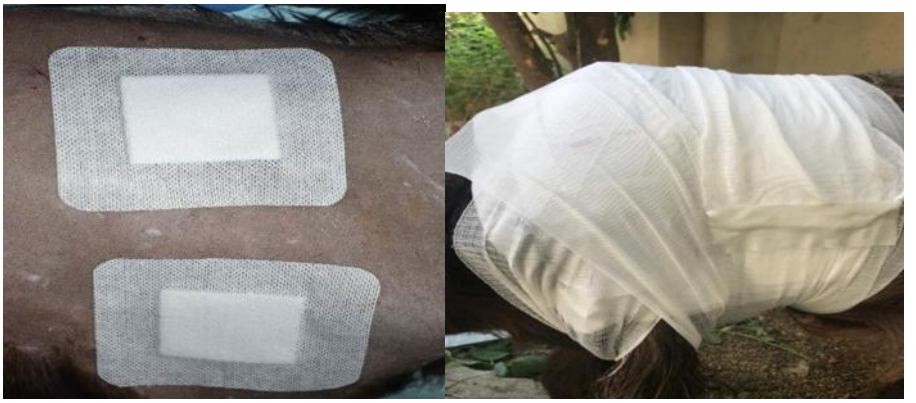


Figure 4. Bandaging adopted for wound protection and coverage in between treatments

Results

Histopathological observations

The effects of CFDAM with NTP on wound healing were studied using a full-thickness skin wound model in bucks. The results of histopathological observations at different time intervals are presented in figures at table 2,3. The histopathological observation scores in different groups at different time intervals are presented in table 1, according to (sultana *et al.,* 2009), to analyze wound healing at the microscopic scale, Hematoxylin and Eosin (H&E) or mason's Trichrome, tissue staining was performed, two-way ANOVA used for data analyzes between groups and days. In group I on day 3, showed severe inflammatory reaction, plenty of neutrophils infiltration and fibrin networks deposition in incision gap under necrotic tissues, vertical orientation reticular pattern collagen fiber was seen with profound- granulation tissues.in the same group and time sections stained with at3days post incision by mason's trichrome stain was neutrophils infiltration and fibrin networks under necrotic tissue with moderate light blue collagen fiber vertical orientation and reticular pattern of collagen fiber infiltrated with severe inflammatory cells, the total histopathological score was 4 at this stage.

In group II on day 3, showed 2 to 3 epithelial cells extended over granulation tissue and under tissue debris, the incision gap filled with profound vascular granulation tissue vertical orientation infiltrated with plenty of inflammatory cells under marked cellular debris, by Mason's trichrome stain shows the incision gap filled with moderate vascular granulation tissue with moderate light blue collagen fiber vertical orientation and reticular pattern of collagen fiber infiltrated with moderate inflammatory cells, the total histopathological score was 7. In group III on day 3, there was epithelial cells 2 to 3 cells thickness extended under tissue debris, moderate vascular granulation tissues with vertical orientation, reticular pattern of collagen fiber and moderate infiltration with neutrophils and mononuclear cells, the total histopathological score was 7.83 at3 days post incision by Mason's trichome stain was thin 2 to 3 cells thickness of epithelial cells extended over moderate vascular granulation tissues light blue

color moderate collagen fiber infiltrated with neutrophils and mononuclear cells and under cellular debris. The inflammatory infiltration was the same in treatment groups in comparison with control group the inflammatory infiltration was increased significantly (P< 0.05), the amount of granulation tissue was increased in treatment groups in comparison with control group with significant difference (P< 0.05). Also, the collagen fiber orientation and the collagen pattern in wound bed histology at 3 days, was the same in all groups. Moreover, the amount of early collagen, in II treated group was increased significantly (P< 0.05) While in III treated group was high significantly (P< 0.05), in comparison to control group. While amount of mature collagen, was absent in all groups.

In group I On day 7, there was profound vascular granulation tissue with plenty of neutrophils infiltration and vertical collage fiber orientation and reticular pattern and fibrin networks with red collagen fiber fill the incision under necrotic tissue, at 3days post incision by mason's trichrome stain shows the incision gap filled with moderate vascular granulation tissue with moderate light blue collagen fiber vertical orientation and reticular pattern of collagen fiber infiltrated with moderate inflammatory cells, the score was 5.5. In group II On day 7, shows thickness, over 4 cells, epithelial cells extended over moderate granulation tissue with mixed orientation and mixed collagen pattern as well as few inflammatory cells and under cellular debris, at7days post incision by Masson trichrome stain shows the incision gap filled with moderate granulation tissue deep blue color of moderate mature collagen fiber orientation and moderated mixed pattern of collagen infiltrated with moderate of inflammatory cells and the score was 10.83.

In group III on day 7, there was epithelial cells 3 to 4 cells thickness extended over moderate vascular granulation tissue characterized by reticular orientation mixed pattern of collagen fiber moderate infiltrated with inflammatory cells under tissue debris. The histopathological score was 12.5, at7 days post incision by Masson trichrome stain was epithelial cells 3 to 4 cells thickness extended over moderate vascular granulation red color fiber tissue with moderate infiltrated with inflammatory cells. The inflammation decreased significantly (P< 0.05), in II treated group and high significantly (P< 0.05) in III treated group in comparison with control group. Also, amount of granulation tissue, in I and II groups was decreased, while III group was increased with non-significant difference (P< 0.05). The collagen fiber orientation was non-significantly in both I and II group, While in III group was significant difference (P< 0.05). The collagen pattern was non-significantly in I group, while in both II and III group was significant difference (P< 0.05). As well, mount of early collagen, in both II and III group was significantly (P< 0.05) in comparison to I group, and increased in both I and II group. Moreover, amount of mature collagen, was in the same level in I groups at this time and increased in both II and III group was significantly (P< 0.05) in comparison to I group.

In group I On day 21, shows the incision gap filled with moderate vascular granulation tissue mixed orientation and mixed collagen pattern with moderate inflammatory cells infiltration under necrotic debris shows moderate thickness of epithelial layer over red granulation tissue filled the incision gap, at 21 days post incision by Masson Trichome stain was epithelial layer extended over pale green granulation tissue, the total histopathological score was 10.5 at this stage. In

group II On day 21 post incision shows full thickness of epithelial cells with rete ridge over scanty granulation tissue, horizontal orientation, fascicle pattern collagen fiber with few inflammatory cells' infiltration and under tissue debris, the histopathological score was 15.5, at 21days post incision by Masson trichrome stain shows the incision gap filled with moderate granulation tissue with light blue minimal of early collagen fiber, deep blue color of minimal mature collagen fiber horizontal orientation fascicle pattern collagen fiber.

In group III On day 21, was thickness epithelial layer extended over scanty granulation tissue characterized by few congested blood vessels infiltrated with scanty inflammatory B cells horizontal orientation mixed pattern of collagen fiber, the histopathological score was 16.83, at21 days post incision by mason's trichrome stain was full thickness of epidermis over scanty granulation tissue characterized by few congested blood vessels infiltrated with scanty inflammatory cells horizontal orientation mixed pattern of collagen fiber deep blue color in deep incision gap and light blue color of moderate early collagen fiber. The inflammation decreased significantly ($P< 0.05$), in both II and III treated group in comparison with control group. Also, amount of granulation tissue decreased in all groups with significant difference ($P< 0.05$) in treated III group in comparison with control group. The collagen fiber orientation was significantly in I group, While in III and II group was high significant difference ($P< 0.05$). The collagen pattern was significantly in I group significant difference ($P< 0.05$), and III group was high significant difference ($P< 0.05$), while in II group was non-significantly. As well, mount of early collagen, in both II and III group was non-significantly ($P< 0.05$) in comparison to I group, and non-significant difference ($P< 0.05$) in I group. Moreover, amount of mature collagen, was in the same level in II group at this time and increased in both I and III group, in II group was significantly ($P< 0.05$), and in III group was high significant difference ($P< 0.05$) in comparison to I group.

In group I On day 35, 3 to 4 cells thickness of epithelial cells extended under necrotic debris and the score 14.16, the incision gap filled with scanty granulation tissue moderate infiltrated with inflammatory cells and moderated vascular granulation tissue with horizontal orientation and mixed collagen fiber pattern under necrotic debris, By mason's trichrome stain shows the incision gap filled with mixed moderate granulation tissue orientation and mixed matured deep blue collagen fiber pattern infiltrated with moderate inflammatory cells. In group II on day 35 post incision shows full thickness of epidermal l cells with rete ridge over scar tissue with absent of granulation tissue infiltrated with few inflammatory cells, horizontal tissue orientation, fascicle tissue pattern, by mason's trichrome stain shows full thickness of epidermal layer with rete ridge cover by keratin layer over deep blue color minimal mature collagen fiber and absent early collagen fiber and the total histopathological was 17.66.

In group III on day 35, was thickness epidermis with keratin layer over scanty granulation tissue with fascicle pattern horizontal fiber orientation with moderated mononuclear cells infiltration at35 days post incision by mason's trichrome stain was thickness epiderma layer with keratin over scanty granulation tissue deep blue color of moderate mature collagen fiber. The histopathological score was 20 at this stage. The inflammation decreased

significantly (P< 0.05), in both II and III treated group in comparison with control group. Also, amount of granulation tissue decreased in all groups with significant difference (P< 0.05) in treated III group in comparison with control group. The collagen fiber orientation was significantly in all group was high significant difference (P< 0.05). The collagen pattern was non-significantly in I group significant difference (P< 0.05), and in both II and III treated group was high significant difference (P< 0.05). As well, mount of early collagen, in both II and III group was high significantly (P< 0.05) in comparison to I group, and non-significant difference (P< 0.05) in I group. Moreover, amount of mature collagen, was in the same level in II group at this time and increased in both I and III group, in II group was significantly (P< 0.05), and in III group was high significant difference (P< 0.05) in comparison to I group.

Table 1
Effect treatments on inflammatory infiltration, amount of granulation tissue, collagen fiber orientation, pattern of collagen, amount of early collagen, amount of mature collagen of bucks[1]

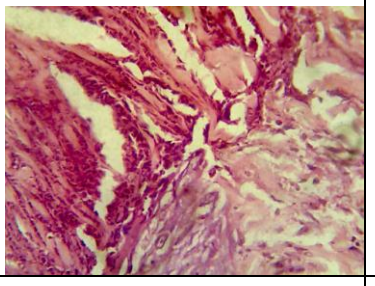
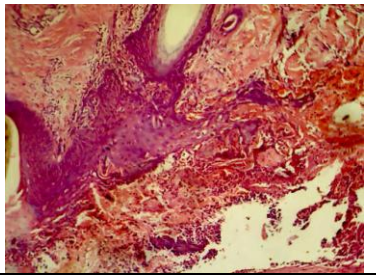
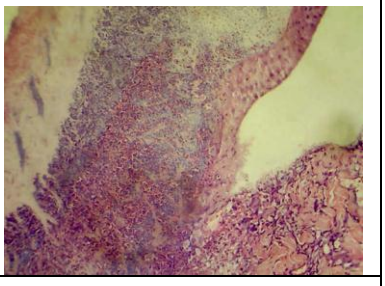
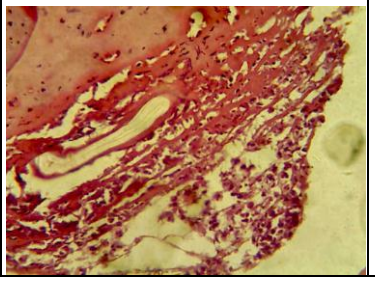
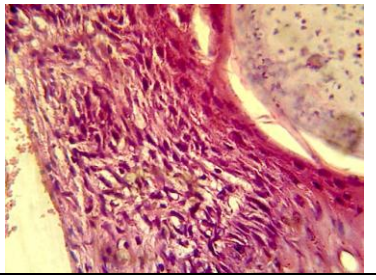
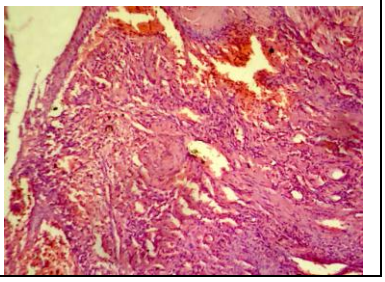
Treatment							
Time	Group	IF	AGT	CFO	PC	AEC	AMC
3	(group I)	1.00c	1.00d	1.00c	1.00c	0.00e	0.00d
	(group II)	2.00b	1.83c	1.00c	1.00c	1.00d	0.00d
	(group III)	2.00b	2.00c	1.00c	1.00c	2.00a	0.00d
7	(group I)	1.00c	1.17d	1.00c	1.16c	1.00d	0.00d
	(group II)	2.00b	2.00c	1.17c	1.83b	2.00c	2.00b
	(group III)	2.83a	2.00c	2.00b	1.83b	2.00c	2.00b
21	(group I)	2.00b	2.17c	2.17b	2.00b	1.16d	1.00c
	(group II)	2.83a	2.83b	2.83a	2.00b	2.16c	2.00b
	(group III)	3.00a	3.00b	2.83a	2.83a	3.00b	3.00a
35	(group I)	2.00b	3.00b	2.83a	2.16b	2.16c	2.00b
	(group II)	3.00a	3.00b	3.00a	3.00a	3.66a	2.00b
	(group III)	3.00a	4.00a	3.00a	3.00a	4.00a	3.00a
SEM		0.068	0.096	0.108	0.107	0.103	
Main							

effects							
Group							
(group I)		1.50c	1.83c	1.79b	1.58c	1.083b	0.75c
(group II)		2.46b	2.46b	1.96b	1.96b	2.41a	1.50b
(group III)		2.71a	2.71a	2.21a	2.17a	2.54a	1.96a
SEM		0.034	0.048	0.054	0.053		
Time							
3		1.67c	1.61c	1.00d	1.00d	1.00d	0.00d
7		1.94b	1.72c	1.39c	1.61c	1.66c	1.28c
21		2.61a	2.67b	2.61b	2.28b	2.11b	2.00b
35		2.67a	3.33a	2.94a	2.72a	3.28a	2.33a
SEM		0.039	0.055	0.062	0.062	0.059	
P-Values							
Treatment		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Time		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Group x Time		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a-c] Means within a column note sharing a letter in their superscript significantly different at $P \leq 0.05$

IF= inflammatory infiltration, AGT=amount of granulation tissue, CFO=Collagen fiber orientation, PC= pattern of collagen, AEC= amount of early collagen, AMC=amount of mature collagen

Table 2
Histological observations of all groups on days 3,7,21 and 35 after wound generation. Wound tissue sections were stained with hematoxylin eosin (H&E)

days	control	FCADM	FCADM+NTP
Day 3			
Day 7			

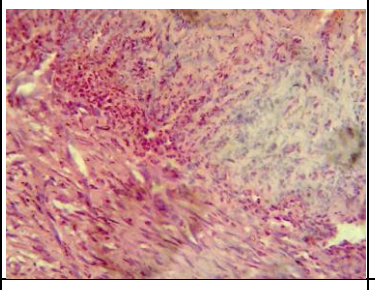
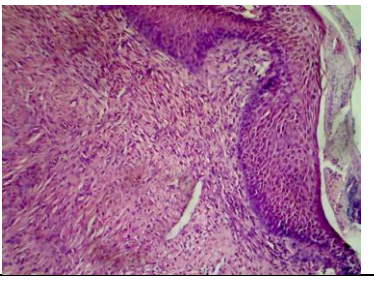
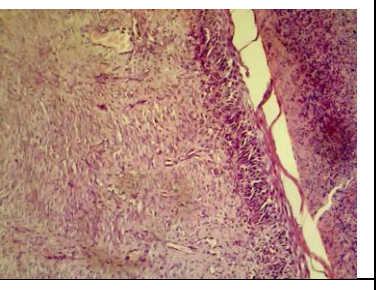
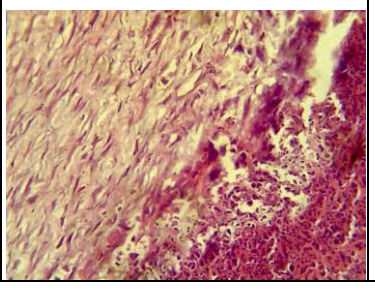
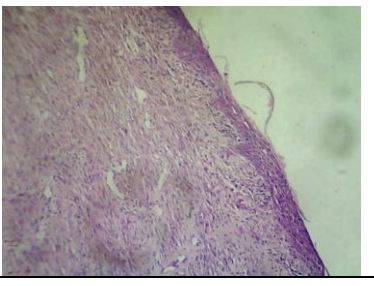
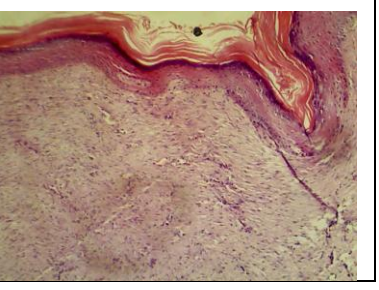
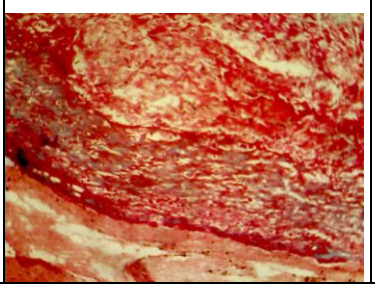
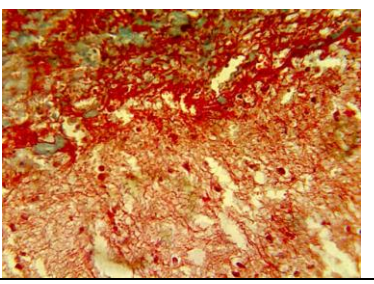
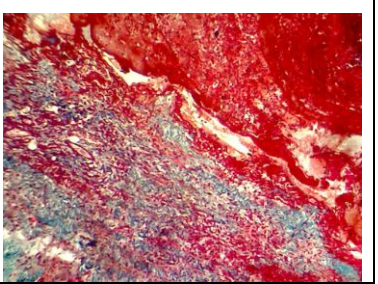
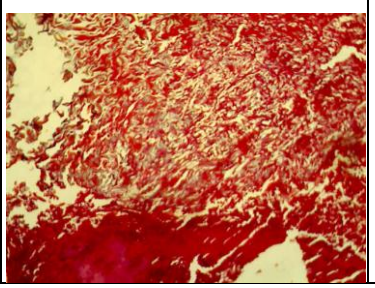
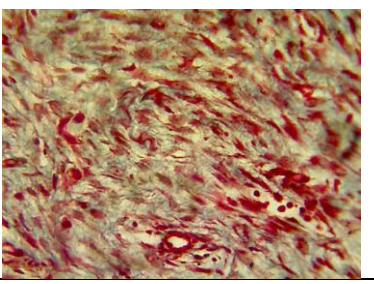
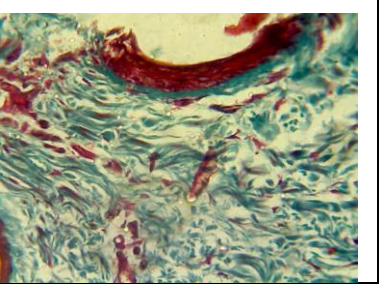
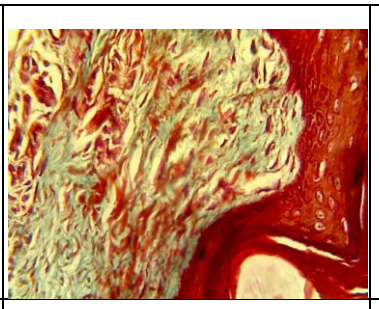
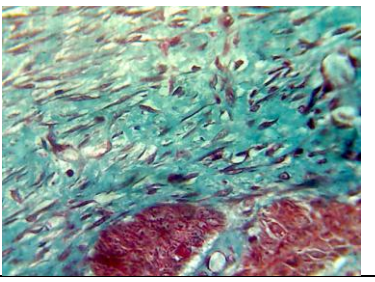
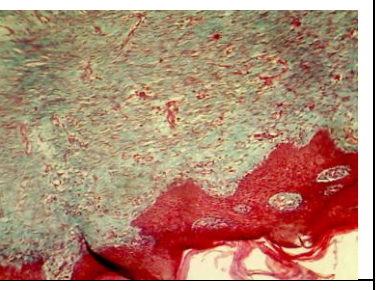
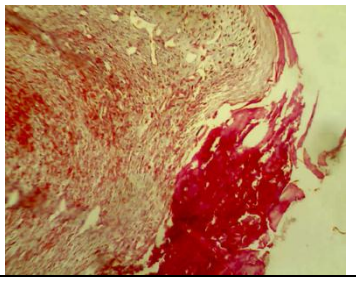
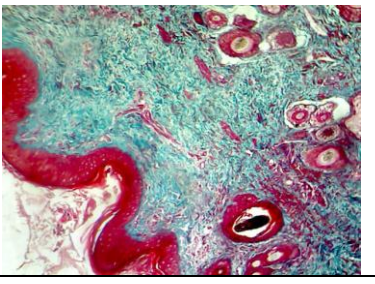
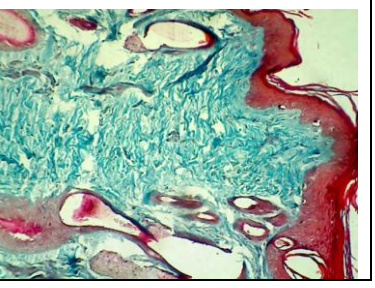
Day 21			
Day 35			

Table 3
Histological observations of all groups on days 3,7,21 and 35 after wound generation. Wound tissue sections were stained with masons trichrome

days	control	FCADM	FCADM+NTP
Day 3			
Day 7			

Day 21			
Day 35			

Discussion

Wound healing comprises a cascade of multiple biological and biochemical processes, which consist of a series of phases involving blood clotting, inflammation, cell proliferation, migration and differentiation, leading to the formation of granulation tissue, wound contraction, and final tissue restoration and remodeling. The key roles in these processes are played by growth factors, cytokines and inflammatory mediators released at the wound site (Isbary et al. 2010). Alterations that disrupt controlled healing processes may then lead to chronic or non-healing wounds, or excessive fibrosis. Based on its non-specific antimicrobial effects, NTPs appear to be a promising biomedical tool for effectively eliminating wound contamination and restarting the normal wound healing process. Indeed, several clinical trials have already approved the potential of NTP in the elimination of bacterial load, as well as for improved chronic wound healing (Shao et al. 2016). In the present study evaluated the efficacy of our NTP system in the healing of full thickness acute skin wounds in bucks' models. While most of the already published studies on wound healing were carried out using argon plasma (Arndt et al. 2013; Schmidt et al. 2017), in this study applied air plasma, which generates high amounts of reactive nitrogen species, such as nitric oxide (NO), nitrogen dioxide (NO2) and reactive oxygen species such as ozone (O3), superoxide (O2−) and hydroxyl radicals (·OH). Our results are in line with the findings of the study by (Yu et al. 2011).

Showed that argon plasma treatment promoted acute inflammation. In particular, inducible nitric oxide synthase (NOS2, iNOS) is an enzyme responsible for the endogenous NO release, which is a highly diffusible intercellular signaling molecule that has been shown to regulate many processes in human skin physiology. The beneficial effects of NO on wound repair may be attributed to its functional influences on angiogenesis, inflammation, cell proliferation, matrix deposition, and remodeling. Indeed, high iNOS levels were associated with higher

healing rates in chronic leg ulcers, while impaired wound repair was associated with lower NO bioavailability. As air plasma treatment is capable of increasing NO accumulation in the living tissue, exogenic NO generated by the plasma may also stimulate NO synthesis through increased activity of iNOS. Enhanced NO concentration in the skin tissue might thus be the key molecular mechanism responsible for the plasma induced acceleration of the healing process, as well as wound closure. On the other hand, overexpression of iNOS may be attributed to excess collagen formations in keloid lesions. The study observed wound contraction, the wound area was significantly more reduced in the bucks treated with NTP than in that of the untreated bucks at day 3 up to 35 days. Respectively, improved wound contraction after the plasma treatment has also been confirmed in other studies using NTP generated from argon, helium or nitrogen (Ying et al. 2011).

This increase is thought to be trigger for the proliferation and migration processes responsible for accelerated healing, and evidence in this respect has been previously obtained in vitro (Arndt et al. 2021). Indeed, through the detection of the collagen it was possible to confirm in vivo the stimulation of cell proliferation, resulting in an accelerated closure of the wound. This result is in agreement with observations performed by several authors about the ability of the plasma treatment to stimulate proliferation of fibroblasts (Neretti et al. 2018; Bekeschus et al. 2016; Liu et al. 2017). In particular, a research group found using total proteome profiling an overall reduction of integrin protein expression in plasma-treated fibroblasts, a condition which is often associated with loss of focal adhesion, increasing cell motility (Arndt et al. 2015). The inflammation reduction observed at a late stage of the healing process, despite not being very strong, was statistically significant. The role of non-thermal plasmas in reducing inflammation, either by modulating the expression of TGFβs or through other pathways, has been indeed reported by other authors (Bekeschus et al. 2015; Choi et al. 2016). This outcome is related to the more general topic of redox control of inflammation (Schafer and Werner 2008; Schmidt and Bekeschus 2018). Of particular interest is the observation that the plasma treatment promotes vascularization at two weeks after lesion induction. Indeed, it is known that a moderate oxidative stress level plays a positive role in angiogenesis (Kim and Byzova 2014).

Chronically produced or highly concentrated ROS are detrimental for most tissues, whereas transient or low levels of ROS are able to activate signaling pathways that eventually promote regeneration and growth of blood vessels (Wong 2017). VEGF is the most potent and primary endothelial specific angiogenic growth factor, both in physiological and pathological conditions. It is to be remarked that, while our study detected angiogenesis through histopathological, several recent studies have identified new mechanisms of ROS-activated angiogenesis that operate in a VEGF-independent manner, and which may have gone undetected in the present study Ngo MHT, (Shao et al. 2014). The capability of plasma treatments to promote angiogenesis has been reported by several groups. In particular, a non-thermal plasma was found to induce pro-angiogenic and angiogenesis-related factors in skin keratinocytes, fibroblasts and endothelial cells, and to promote wound angiogenesis via autocrine and paracrine mechanisms (Arjunan et al. 2011). Also, an enhancement of angiogenesis was

detected through the increase of blood flow and CD31 expression in burn wounds induced in mice (Kisch et al. 2016). Angiogenesis improvement was also measured in association to a Dielectric Barrier Discharge (DBD) treatment and related to the release of Fibroblast growth factor-2 (FGF-2) (Clark 1993). Repetitive DBD treatments were also found to boost cutaneous microcirculatory effects in a prospective, cohort trial (Grinnell 1994). In the same study also enhanced angiogenesis and reduced inflammation at late stage were detected.

Overall, the results of this in vivo study point to an accelerated wound healing, related to the enhancement of the intracellular ROS level, which includes reduced inflammation at late stage of the healing process, a faster reformation of blood vessels and enhanced hair growth. Re-epithelialization plays an important role in wound closure (Schwentker et al. 2002; Stallmeyer et al. 1999). The enhanced NO concentration plays an important role in promoting wound healing (Droge 2002). Delayed re-epithelialization of wounds when NO synthesis is inhibited (Fisher and Rittié 2018). It has been reported that reactive species, including NO, result in positive effects on keratinocytes and the stimulation of angiogenesis (Liebmann et al. 2011). The regeneration of a functional epidermis depends on the reconstitution of the dermal–epidermal junction (Rizzi et al. 2010; Kavros 2012).
CFDAM provides the ideal environment to support dermal tissue generation that contributes to successful healing outcomes with infrequent applications. The matrix is composed of fetal caprine dermis processed to preserve the 3-dimensional architecture of the dermal collagen fiber network and to retain the biochemistry of the fetal dermis. The collagen composition of CFDAM is unique, as it is the only graft that contains physiological amounts of type III collagen found in the extracellular matrix of healing wounds (Gangwar et al. 2013). In addition, the processing technology renders the matrix free of cells, growth factors, and glycosaminoglycans, which can be associated with a strong inflammatory profile and rapid degradation and reabsorption of the matrix (Kavros 2012).

By removing these components, no foreign body reaction occurs to CFDAM, allowing the fetal collagen matrix to persist and act as a dermal scaffold during the initial stages of healing. Upon CFDAM implantation, the animal's cells and growth factors rapidly reconstitute the collagen scaffold and facilitate angiogenesis leading to dermal tissue generation (Strauss and Brietstein 2012) as well as re-epithelialization by wound margin keratinocytes (O'Loughlin et al. 2013; Doornaert et al. 2019). Because the collagen matrix of CFDAM is not degraded or reabsorbed, but rather repopulated and revascularized, only 1 or 2 applications may be required for dermal generation, unlike other advanced wound therapies that require the body to produce the necessary dermal extracellular matrix proteins. The reduced requirements for collagen production and reorganization may explain the decreased time to healing over that of other products. Several studies using ADM on skin wounds have found more pronounced proliferation of blood vessels and fibroblasts within ADM (Hartmann et al. 2013). The blood vessels that proliferate inside the ADM supply nutrients and oxygen for epidermal cell maintenance and proliferation (Schultz and Wysocki 2009). In addition, in the literature, the viability and functionality of the constituent elements of the dermis are well documented to be crucial for the viability and functionality of epidermal cells (Waaijman et al. 2010; Zajicek et al. 2012)ADM behaves as an appropriate

substrate for the adhesion, growth and differentiation of keratinocytes (Chen et al. 2018). The facilitating role of ADM in the migration and proliferation of keratinocytes in skin wounds is confirmed by increasing the gene expression of Keratin 19 (K19) and ß-1 integrin, which are the biomarkers of stem cells of keratinocytes (Goodarzi et al. 2018). A thickened epidermis is directly related to a thickened dermis, and an increase in the constituent elements of the dermis is fundamental for keratinocyte proliferation.

wound healing in skin is highly complex where many types of cells are involved in a multistep process in which numerous cell growth factors are supplied. These growth factors stimulate the formation of granulation tissue and matrix, promoting epithelization, especially the period of proliferation of vascular epithelial cells and endothelial cells, which play extremely important roles. Among them, bFGF, VEGF and PDGF have strong angiogenic effects and are particularly important for promoting matrix formation and promoting cell growth and proliferation 85. It is believed that the expression of bFGF, VEGF and PDGF in skin wounds can directly reflect the ability of biomaterials to promote healing. The masons' trichrome results are consisting with the results of H&E staining. CFADM showed good bioactive properties by promoting the expression of bFGF, VEGF and PDGF which are beneficial for skin wound healing. the CFADM provides an excellent environment for regeneration of the wound. This kind of temporary wound coverage, or corium grafting in a different term, directly takes part in a series of wound repair activities, such as wound healing and maintenance of stable internal environment (Botchkarev and Kishimoto 2003). It can also provide better coverage of the wound. It adheres to the wound surface well. It is free from immunogenicity. The collagen contained in the dermis is capable of inducing cellular regeneration. The specific three-dimensional texture has the function of cellular conglomeration and congregation. Moreover, it could promote the reconstruction, adhesion, differentiation and proliferation of tissue and cells. Therefore, it could facilitate dermis reconstruction, shorten epithelialization time and decrease the probability of hyperplastic scar.

In conclusion, this study has demonstrated that 30 sec NTP and CFDAM treatment promoted healing of skin wounds in bucks, by an improved wound closure in the proliferative phase of the healing. Of note, and similar to many other studies, this study used a model of acute and sterile wound repair, which does not imply distinct pathophysiology of impaired healing in chronic and contaminated wounds. As NTP has bactericidal effects, its primary role in the wound healing process is to inhibit bacterial infection. In addition, in this study showed that NTP can also stimulate wound healing by other indirect mechanisms. CFDAM provides the ideal environment to support dermal tissue generation that contributes to successful healing outcomes with infrequent applications, itis had good biocompatibility and could accelerate the wound healing process.

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