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PLASMA INVESTIGATIONS

Floating Discharge Plasma for Healing of Tendon Injury

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Abstract—In this paper the effect of irradiation of cold plasma on the skin for healing of injured tissue, which is located inside a body (such as tendon), is evaluated. Cold plasma was applied to the rabbits skin after suturing and continued for 21 days. After 21 days the tendon tissue was considered histological. The results show that cold plasma treatment led to reduction of the inflammatory cells and acceleration of the recovery phase by increase neovascularization and collagen production. At the non-touching cold plasma production of ultraviolet, neutral radicals, and electric fields led to some effect on cells. To find the effect of electric fields, ultra violet, and reactive oxygen species, the indirect plasma treatment compared to direct treatment. To produce indirect plasma, a grounded mesh was put as a second electrode. The results of this study confirm that the cold plasma treatment of skin has positive effect on healing of tissue inside a body. The electric fields has no effect on the healing process. Results of this study show that reactive oxygen species is the parameters that causes healing.

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INTRODUCTION

In recent years cold atmospheric plasma become to a new treatment method. Many researchers applied cold plasma for wound healing.

For example, in [1], by a retrospective study it was shown that cold atmospheric plasma could be an effective method especially in chronic wounds. Authors of [2] showed combined treatment of argon and helium could accelerate skin acute wounds healing.

The literature focuses on applying cold plasma on the surface of wound. Not all injuries occur on the skin, some of them occur in tissues located inside a body. For healing of some of this wounds surgery is needed.

Healing of this injuries without surgery is important and could help many patients. In this paper we try to show if skin irradiation by plasma could affect the healing processes of the inside injuries.

For this aim the tendon tissue is selected. Tendon injuries are a common problem during sports, and among soft tissues tendon heals slowly.

Tendon healing includes three stages: inflammatory, a proliferative and remodeling stage, which takes slowly [3]. The main goal for treatment of injured tendons is return to normal tensile strength. Some therapies, such as tissue engineering, gene therapy, physical therapy, and laser therapy were applied for fast healing [4]. In this study, the rabbits were treated by the cold plasma treatment for 30 s.

Paper [5] shows that short treatment time of FE-DBD (floating electrode-dielectric barrier discharge) plasma up to 30 s induces proliferation of endothelial cells, which are responsible for the control of the fluid flow out and into a tissue, but the longer treatment time leads to apoptosis. Authors of [6] showed that 30 s of DBD plasma treatment is required for migration of fibroblasts and the plasma treatment for 2 min leads to cell detachment of the confluent cell layer [6]. In [7], it was reported that the 30-s treatment by DBD plasma leads to improvement of human keratinocyte.

There are no study about tendon healing by cold plasma for comparison. There are no study about the applying cold plasma on skin for treatment of inside wound as well. In this case, the ionization wave does not touch the wound.

Touching plasma could generate charging of surface. In the non-touching cold plasma production of ultraviolet (UV), neutral radicals, reactive oxygen species (ROS), and electric fields led to some effect on cells [8, 9].

The FE-DBD plasma was selected for this study. As FE-DBD applied in ambient air produce high amounts of ROS and reactive nitrogen species [6]. As the current of FE-DBD plasma is less than 5 mA, the plasma is safe [10].

On the next step, for evaluating the effect of UV, electric fields, and ROS in healing of tendon injury, the direct and indirect plasma treatments were compared. The rabbits were treated by indirect plasma and the results of the pathological analysis of the tendon was compared with the results for direct plasma treated

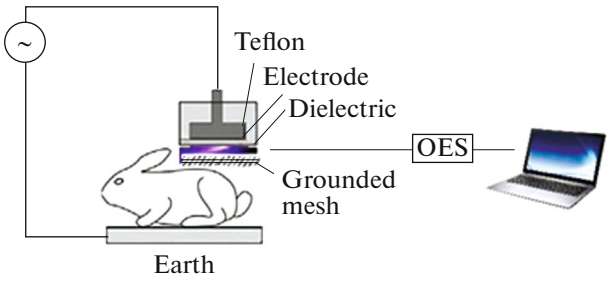

Fig. 1. The scheme of plasma treatment of rabbits (OES—optical emission spectroscopy).

tendon. The indirect plasma was created using meshes as ground electrode in the setup (according to Fig. 1).

Treatment of direct plasma includes flux of charges (electrons, positive and negative ions) and active uncharged species, UV.

Indirect plasma generates uncharged atoms and molecules but no charged particles. Therefore, the effect of the charged particle of cold plasma and electric fields were omitted.

MATERIALS AND METHODS

Cold Plasma Setup

FE-DBD setup is driven by 10 kV and 10 kHz AC high voltage applied between the electrode and skin of the rabbit. The circular copper electrode (radius is 2 cm) was covered by dielectric. The rabbits were treated by cold plasma. For producing indirect plasma, a grounded mesh was used as a second electrode (Fig. 1).

The UV power density was determined using HAMAMATSU UV power-meter. The voltage and current were determined using a Tektronix P6015A high-voltage probe and current probe (TCP-202). The waveforms of current and voltage were recorded by a Tektronix DPO digital oscilloscope. The temperature of cold plasma was determined using Boltzman plot method:

$$TE = \frac{E_k - E_i}{K}\left[\ln\left(\frac{A_k g_k I_i \lambda_i}{A_i g_i I_k \lambda_k}\right)\right]^{-1},$$

where λ is wavelength of the emitted light; A_j is transition probability, $j = k$, i; E_j is upper energy level; g_j is statistical weight of the state.

Design of the Experiment

Eighteen healthy rabbits were purchased from the Pasteur Institute of Iran. All of them were male, white New Zealand, (20 weeks old) and the body weight of them was about 2.5–3.5 kg. The rabbits were divided into three groups of six rabbits each: control group—animals with tendon injuries without any treatment; groups two and three—animals with tendon injuries were treated by the indirect or direct cold plasma). At first anesthesia was induced with combination of xylazine hydrochloride 2% (8 mg/kg) and ketamine hydrochloride 10% (35 mg/kg). After anesthesia and clipping, the rabbits' skin was disinfected by antiseptic povidone-iodine solution. The skin (2 cm) was cut and the right tendons were freed from surrounding tissues. Then the sharp incision was made. The skin and tissue were sutured. Cold plasma was applied to the rabbits' skin after suturing and continued for 21 days. The rabbits were treated by cold plasma 30 s each day (according to Fig. 1). The distance between the rabbit and dielectric plate was 1 cm. The rabbits were kept in standard condition: standard cages, filtered tap water, standardized food, room temperature 18–22°C, humidity of 40–50%, and 12 h/12 h light/dark cycle. After 21 days the tendons were analyzed histologically. At first tendons were washed in physiological solution then they were fixed using 10% formalin and embedded in paraffin. The sections were kept under light microscopy. The inflammatory phase, production and arrangement of collagen were considered with a specific grading as follows: (0) no, (1) mild, (2) intermediate, and (3) severe.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 18). One-way analysis of variance (ANOVA) followed by Tukey post-hoc test were employed to analyze the results. All statistical tests were performed at a significance level of $P < 0.05$. Results were expressed as mean standard deviation.

RESULTS AND DISCUSSION

Effect of Cold Plasma on Histological and Mechanical Parameters

The results of this study (Fig. 2) indicated a lower rate of healing in control groups in comparison with FE-DBD plasma treatment. The optical emission spectroscopy of the FE-DBD plasma (Fig. 3) showed the emission species. Figure 4 shows the current and voltage curves of the plasma. The voltage and current of the plasma were 10 kV and 3 mA, respectively. The temperature of the plasma was 1.8 eV and the density was 5×10^{15} cm^{-3}. The UV power densities of direct and indirect plasma were 0.2 and 0.09 mW/cm^2.

Tendon repair divided into three main phases: inflammatory phase, a proliferative or repair phase, and a matrix remodeling phase [3]. The sampling results (Fig. 2) indicated that formation of the epithelium increased during healing, which confirms the success of the diagnosis. The inflammatory cells were of significantly lower concentration in the tendon treated with cold plasma than in control ones, which confirms that cold plasma treatment reduce the inflammation phase. Cold plasma treatment resulted in reduction of inflammatory phase and acceleration of the recovery phase by increase of neovasculariza-

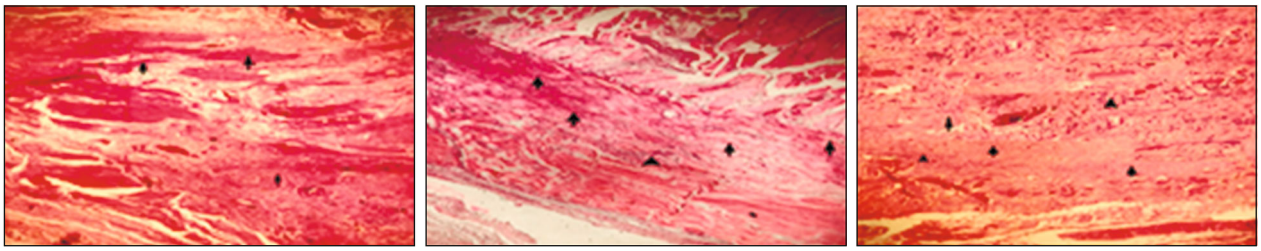

Fig. 2. Histophatological images of tendon after 21 days: (a) control group, (b) direct plasma, and (c) indirect plasma groups.

tion and collagen production. Figure 2 shows that after the cold plasma treatment the strings of collagen have become more regular and thicker, which indicates improvement. The neovascularization is higher in the cold plasma treated group.

There are no study about tendon healing by cold plasma for comparison. Also there are no study about the applying cold plasma on skin for treatment of an inside wound.

The cold plasma treatment resulted in reduction of inflammation phase. Inflammation response is a protective phase that starts the process of tendon repair. At this phase, generation of excessive nitric oxide led to increase the production of ROS [11].

ROS induces oxidative stress, which causes lipids, proteins, and DNA damages and fibrosis [12–15]. Some reports showed that low-level laser therapy has positive effect on tendon healing [16, 17]. It has been demonstrated that low-level laser therapy could enhance ROS. There are number of reports saying that ROS plays an important role in the inflammatory response [11]. This shows that the healing process in the plasma group was faster than in the control group. Increasing trends in the plasma group was quicker, and always one step ahead of the control group, which indicates that plasma improved the healing compared to the control group due to the inhibition of the infection by helium plasma. Collagen formation in the helium plasma group was always faster than in the control group, which illustrates the positive effects of the plasma in treating tendon. Recent reports show that low-level laser therapy accelerate tendon healing by increasing collagen types I and III [3, 18].

In this study, cold plasma treatment of the tendon resulted in increased of production of collagen. Production of collagen is important to the repair strength of tendons [19]. One of the primary factors responsible for the slow healing of a tendon is poor blood circulation, which leads to low levels of oxygen and nutrients in the region of the wound [20]. Neovascularization is another important factor in the healing of a wound [21]. The results show that plasma treatment increases the neovascularization of the wounds as compared to the control group. Authors of [22] hypothesized that the reactive particle present in plasma can cause the activation of growth factors accomplished by angiogenesis. The results also indicate that the keratinocyte cell migrates in case of wounds treated with cold plasma after a few days of the treatment; this is not

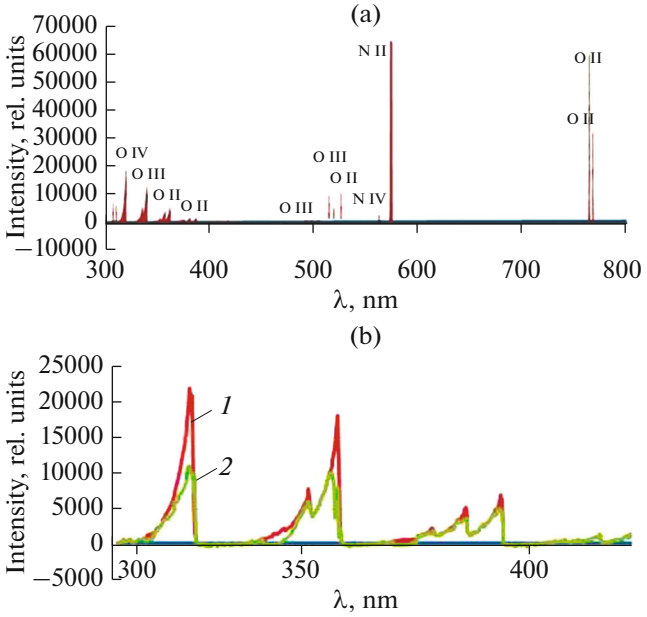

Fig. 3. The optical emission spectroscopy of the FE-DBD plasma: (a) the optical emission spectroscopy of the FE-DBD plasma without mesh; (b) OES analysis of FE-DBD plasma with mesh.

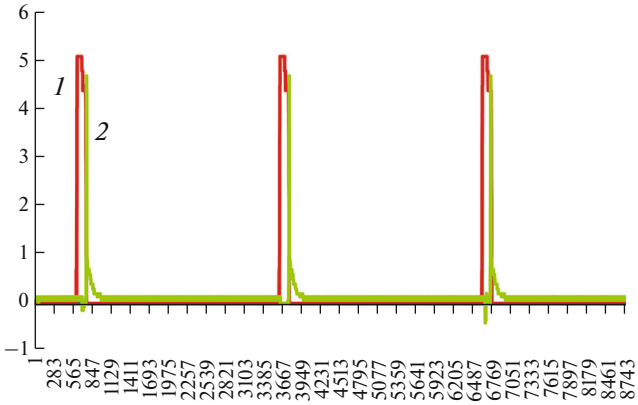

Fig. 4. The current (*1*) and voltage (*2*) curves of the plasma.

observed in the rabbits of the control group. Plasma produces certain species, such as free radicals. Reactive ions and radicals play an important role in the interaction between the plasma and cells. It was reported that NO activated the TGF-β 1 cytokine by S-nitrosation of the latency-associated peptide and the MAPK pathway [23]. The MAPK pathway is known to play an important role in the healing of wounds as well as to be involved in the cellular inflammation-proliferation process. In [24], it is reported that reactive free radicals are involved in the angiogenesis process by activating the vascular endothelial growth factors. In addition, reactive free radicals are responsible for the sterilization of the wounds [24]. In this study, cold plasma treatment on skin led to reduction of the inflammation phase and acceleration of the recovery phase in tendon tissue inside a body. This study demonstrated that the effect of cold plasma is not limited to the irradiated surface.

In these terms, the ionization wave does not touch the wound. Touching plasma could generate charging of the surface. In the non-touching cold plasma production of UV, neutral radicals, and electric fields led to some effect on cells [8, 9].

Some researchers show that electric fields could affect the cells [25, 26]. It has been demonstrated that spreading of ionization waves led to delivery of electric filed, which could penetrate in mammalian tissue about some mm deep [27].

The pervious researches show that the electric filed cause permeability of mitochondria membrane, activate MAPKs pathways apoptosis of *T* cells [28–30], signals muscle contraction by movement of calcium and potassium, nerve signal spread through axon, or restore cell balance [28, 29, 31].

For evaluating the effect of charge and uncharged particles, UV and electric fields in healing of tendon injury, we compare the effect of direct and indirect plasma treatment. Treatment of direct plasma includes flux of charges (electrons, positive and negative ions) and active uncharged species, UV, and electric filed.

But indirect plasma treatment has no charged particles and the UV radiation or the electric field is too low to effect the cells.

Paper [31] shows that in indirect plasma treatment the electric fields are below a few kV cm^{-1}, which is too low to induce significant effects on tissue.

As can be seen from the results in Fig. 3, the UV radiation of the indirect plasma is less than of direct plasma. It was about 20–40% reduction in UV peaks. These results are in accordance with the last literature [32].

The results in Fig. 2 shows that there are no significant difference between healing of tendon tissue with direct and indirect treatment. The scores of healing in both treatments are the same. At first, it has been concluded that the indirect plasma treatment is an effective treatment.

Authors of [33] applied indirect cold plasma on a sheep surgical wound. They reported that the plasma treatment led to reduction of inflammation, increase in neovascularization, cutaneous adnexa, and cell proliferation. Also it has a positive effect on regrowth of hair.

In [5], it is shown that less proliferation of mammalian breast epithial cells is due to the ROS production of indirect plasma treatment.

As the effect of charged particles, electric fields, and UV radiation is omitted in indirect plasma and the healing effects of the both direct and indirect plasma treatments are the same. It can be concluded that ROS and uncharged species of cold plasma are responsible for the healing of injured tissue inside a body.

CONCLUSIONS

The cold plasma treatment had shown to be an effective way for tendon injury treatment.

The results show that the cold plasma treatment resulted in reduction of inflammatory phase and accelerate the recovery phase by increase neovascularization and collagen production. The results of this study confirm that the cold plasma treatment of skin has positive effect on healing of tissue inside a body. Comparison of the effects of direct and indirect cold plasma showed that ROS and uncharged species of cold plasma are responsible for the healing of injured tissue inside a body.

COMPLIANCE WITH ETHICAL STANDARDS

The experiment of this study was done according to the guidelines of the animal care approved by the ethics committee in Islamic Azad University.

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