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Research Article

Effect of Culture Medium Treated with Non-thermal Plasma Energy on the Growth and Viability *In-vitro* of Fibroblast Cells from Asian Elephants *(Elephas maximus)*

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Abstract

Non-thermal plasma (NTP) is being developed for a wide-range of medical applications such as improvement of wound healing, elimination of infective microorganisms, and treatment of cancer. This study investigated the effect of culture medium exposed to NTP on the proliferation in-vitro of skin fibroblasts from Asian elephants. Dulbecco's Modified Eagle's Medium (DMEM) was used as culture medium and was exposed to NTP with three different intensities. The NTP reactive species Nitrite (NO^{2-}) was measured in the treated medium before addition to cells. Fibroblasts were incubated for 24 h with NTP-treated complete medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic/antimycotic. Cell proliferation, the number of cells and viability rate were analysed using flow cytometry 24, 48 and 72 h after the start of the incubation. The proliferation rate of fibroblasts incubated with NTP treated medium was significantly higher ($P<0.05$) than controls and increased in a dose-dependent manner with increasing amount of NTP. Incubation of fibroblasts with NTP did not reduce their viability even at the highest dose of NTP. Culture medium treated with NTP energy may be used to improve healing of skin wounds in elephants. This study successfully shows that the medium treated with NTP was able to stimulate elephant skin fibroblasts proliferation and increase the total cell count but did not reduce cell viability in vitro. Containing buffering agent in culture media might reduce the effect of ROS generated by NTP. This might prevent using high dose of NTP to cause cell apoptosis and induce cell necrosis in this study. Future studies on the skin of living elephant are encouraged to develop more effective and optimum treatment conditions.

Keywords: *Asian elephant, Non-thermal plasma, Culture, Fibroblasts, Skin*

Termal Olmayan Plazma Enerjisi Uygulanan Besiyeri Ortamının Asya Fillerinin *(Elephas maximus)* Fibroblast Hücrelerinin Büyüme ve Canlılığı Üzerine *In-vitro* Etkisi

Öz

Termal olmayan plazma (NTP), yara iyileşmesinin hızlandırılması, enfektif mikroorganizmaların eliminasyonu ve kanser tedavisi gibi çeşitli tıbbi uygulamalar için geliştirilmektedir. Bu çalışmada NTP'ye maruz burakılan besiyeri ortamının Asya fillerinden elde edilen deri fibroblastlarının in vitro proliferasyonu üzerindeki etkisi araştırıldı. Bu amaçla Dulbecco'nun Modifiye Eagle's Medium'u (DMEM) besiyeri olarak kullanıldı ve üç farklı yoğunlukta NTP'ye maruz bırakıldı. NTP reaktif türleri Nitrit (NO^{2-}) hücrelere eklenmeden önce uygulandığı besiyerinde ölçüldü. Fibroblastlar, 24 saat boyunca, %10 Fetal Sığır Serumu (FBS) ve %1 antibiyotik/antimikotik içeren NTP ile muamele edilmiş besiyerinde inkübe edildi. İnkübasyonun başlamasından 24, 48 ve 72 saat sonra hücre proliferasyonu, hücre sayısı ve canlılık oranı akış sitometrisi kullanılarak analiz edildi. NTP ile muamele edilmiş besiyerinde inkübe edilen fibroblastların proliferasyon hızı, kontrol grubundan anlamlı olarak daha yüksek bulundu ($P<0.05$) ve artan NTP uygulaması ile birlikte doz bağımlı olarak arttığı belirlendi. Fibroblastların NTP ile inkübasyonu, en yüksek NTP dozunda bile canlılık oranını azaltmadı. Fillerde cilt yaralarının iyileşmesini hızlandırmak için NTP enerjisi uygulanan besiyeri kullanılabilir. Bu çalışma, NTP uygulanan besiyerinin fil derisi fibroblastlarının proliferasyonunu uyarabildiğini ve toplam hücre sayısını arttırdığını, bununla birlikte *in-vitro* olarak hücre canlılığını azaltmadığını göstermiştir. Besiyerinin tampon madde içermesi, NTP tarafından üretilen Reaktif Oksijen Türlerinin etkisini azaltabilir. Mevcut çalışmada bu durum hücre apoptozisine neden olmak ve hücre nekrozunu indüklemek için yüksek NTP dozlarının kullanılmasını engelleyebilir. Daha etkili ve optimum tedavi koşullarının geliştirilmesi için canlı fillerin derisinde yapılacak çalışmalar teşvik edilmelidir.

Anahtar sözcükler: *Asya fili, Termal olmayan plazma, Besiyeri, Fibroblastlar, Deri*

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INTRODUCTION

Wounds to the skin of Asian elephants *(Elephas maximus)*'s are a common health and welfare problem [1]. Physical damage to the skin of elephants frequently results in wounds. Other causes of skin wounds may include sunburn and nutritional imbalances. Elephant's skin wounds are particularly susceptible to infection and heal slowly because the skin is thick (2.0-2.5 cm) and without sebaceous glands. Compared with other mammals the skin of elephants is dry and takes longer to heal [1]. Clinical treatment of elephant skin wounds leads to a higher risk of drug resistance and a high cost for effective drugs. Consequently, there is on-going pressure to find improved treatment for elephant skin wounds.

By definition, non-thermal plasma (NTP) is partially ionized gas where the energy is stored mostly in the free electrons and the overall temperature remains low. The use of NTP has been developed as a medical option to treat wounds in humans [2]. NTP generates reactive oxygen species (ROS), reactive nitrogen species (RNS), UV-radiation, and electric fields [3]. Most of NTP's effects on the biological system are related to reactive species including H_2O_2, O_3, O_2^-, NO, NO_2, N_2^-, and OH which may enhance wound healing [4-6]. It is known that reactive species, free radicals and some ground state molecules, are produced by mammalian immune system cells, macrophages and neutrophils, and can defend against bacteria and viruses, and also regulate cellular functions [7,8]. Furthermore, reactive species are involved in the regulation of signalling pathways such as growth factors and cytokine receptors [9]. In physiological processes, reactive species play a role in the regulation of vascular contraction, blood coagulation, angiogenesis, inflammation, immune system response, and nerve impulse transmission. Also at cellular level, reactive species regulate cell differentiation, division, migration, and apoptosis by controlling cell-to-cell adhesion, biosynthesis of growth factors, and collagen production [9].

Non-thermal plasma has been used effectively in other medical fields such as to remove dental biofilms, to eliminate oral pathogens, to induce apoptosis of malignant cells [10], inhibit the growth of cancer cells [11] and to stop bleeding [12-14]. NTP may promote wound healing and tissue regeneration by increasing fibroblast proliferation by the release of growth factors such as fibroblast growth factor-7 [15]. In recent years, NTP has been described as an effective wound therapy because it has antimicrobial activity [12,16], it reduces inflammation [12,17], promotes wound healing [16] and does not kill eukaryotic cells [18,19]. There have been many studies of the effects of NTP on wounds in humans [16,20-29], and animals such as rats [30,31], mice [32-34] and pigs [35], but no record of application in elephants. The direct use of NTP for the treatment of wounds in elephants is difficult in field practice because of the difficulty of moving and restraining such large animals. Alternatively, using media treated with high energy NTP to treat skin wounds in elephants may be safer and more convenient for veterinary professionals and animal handlers. For experiment on living elephants might be difficult in terms of collecting optimal data from uncontrollable of wound size, location and type on each elephant. Therefore, it is hard to acquire elephant wounds that have similar cause, size, location as well as type of wound. Creating wound for the study in elephant is hardly possible especially in captive elephant. Apart from that, controlling wound hygiene in elephants is rather difficult due to the elephant behaviour to play sand or dirt.

In recent studies, NTP treatment has successfully been used to decontaminate water samples containing biological and chemical agents [36-39]. Furthermore, NTP treated culture media had an effect on cell growth and morphology in cell culture model [40]. When liquid is exposed to NTP, radical species from NTP outflows into liquid and induce chemical change which causes the treated liquid to then have NTP effects which are almost similar to those caused by direct exposure [41]. Our hypothesis is that culture medium treated with NTP will generate ROS that will enhance the proliferation of skin fibroblasts from Asian elephants.

MATERIAL and METHODS

The reagents, the main components of reagents and the medium formulations together with their vendors and concentrations used in this study are listed in *Table 1*.

Isolation and Culture Elephant's Primary Skin Fibroblast Cells

Skin fibroblast cells were obtained from the ear skin of three Asian elephant carcasses (age range 35 to 55 years old) within 24 h of death. Skin samples, size 3×3 cm^2, were brought to the laboratory in transport medium at 0-4°C within 3 h after collection. Ear skin fibroblasts were prepared using the protocol described by Siengdee et al.[42]. In brief, skin tissue samples were cleaned in phosphate buffered saline (PBS) and chopped into small pieces. Precipitated skin tissues were digested with collagenase type II solution and cultured with explant medium containing 20% FBS at 37°C and 5% CO_2. From the 3rd passage onwards fibroblasts were cultured in culture medium containing 10% FBS and 1% antibiotic/antimycotic at 37°C and 5% CO_2 and routinely trypsinized with 0.25% trypsin/EDTA. Fibroblasts at 2nd-4th passage were frozen in liquid nitrogen for long-term storage and passages 2nd to 6th were used for this study *(Fig. 1)*.

NTP Treatment

Non-thermal plasma experiments were performed using atmospheric pressure NTP dielectric barrier discharge (DBD) TS200 (Engineering Production Equipment Medical, Firenze, Italy) with a DBD helium jet direct plasma probe with an overall 22 mm diameter containing an internal

Table 1. *Components of reagent and medium formulations*

Name	Components	Vendor and Stock Concentration
Culture medium (1.000 mL)	dH_2O + DMEM powder + 4.767% Hepes (w/v) + 3.75% Sodium bicarbonate (w/v) + 1 × antibiotic/antimycotic	• Dulbecco's modified Eagle's medium; DMEM (Gibco; Thermo Fisher Scientific) • Antibiotic/antimycotic (100X) (GibcoTM; Thermo Fisher Scientific, Waltham, MA, USA) stock concentration contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B • Heat-inactivated fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) • Collagenase type II (Sigma- Aldrich, St. Louis, Mo, USA) stock concentration contains 1 mg/mL of collagenase II in DMEM w/o serum • Trypsin-EDTA (0.5%), no phenol red (Gibco; Thermo Fisher Scientific) • Phosphate-buffered saline (PBS) (10×) (Gibco; Thermo Fisher Scientific) • Gentamicin (50 mg/mL) (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) • HEPES (Sigma-Aldrich, St. Louis, Mo, USA) • Sodium bicarbonate (Sigma-Aldrich, St. Louis, Mo, USA) • MTT (Bio Basic Inc, Markham, ON, Canada) • DMSO (Sigma-Aldrich, St. Louis, Mo, USA) • N-(1-Naphthyl)ethylenediamine dihydrochloride; NAD 2HCl (Fisher Scientific, Loughborough, Leicestershire, UK) • Phosphoric acid (Carlo Erba Reagents, Barcelona, Spain)
Complete medium	Culture medium + 10% FBS + antibiotic/antimycotics w/o NTP treatment	
NTP-treated complete medium	Culture medium + 10% FBS+antibiotic/antimycotics w/NTP treatment	
Explant medium containing	Culture medium + 20% FBS + 1 × antibiotic/antimycotic	
Transportation medium	Culture medium + 10×antibiotic/antimycotic	
Collagenase solution	Culture medium + 10% collagenase type II (v/v) + 10 × antibiotic/antimycotic	
Washing with PBS	PBS + 10×antibiotic/antimycotic + 50 µg/mL gentamicin	
Routine antibiotic dose	1 × antibiotic/antimycotic (containing 100 units/mL of streptomycin, 100 units/mL of penicillin and 0.25 µg/mL of amphotericin B)	
MTT solution	Culture medium + 0.5% MTT (w/v)	
0.25 % trypsin/EDTA	PBS + 0.25% trypsin/EDTA	
Colour reagent solution	dH_2O + 10% 85% H_3PO_4 (v/v) + 0.1% NAD 2HCl (w/v)	

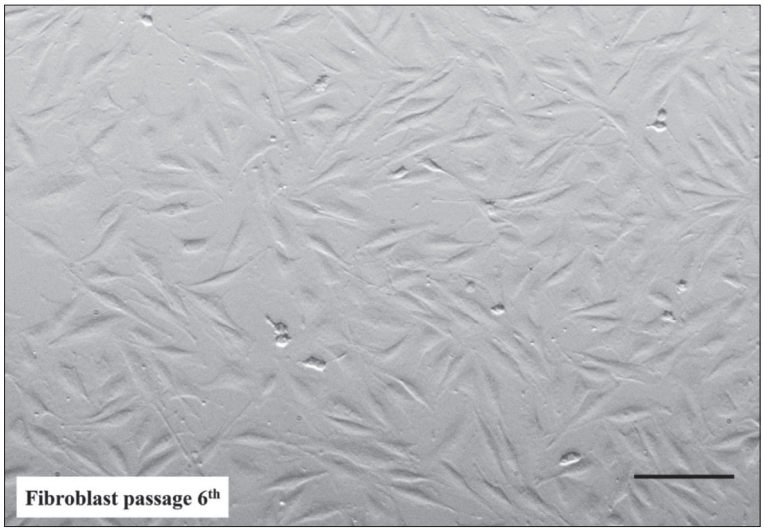


Fig 1. Elephant's skin fibroblast cells used for this study. Scale bar = 20 mm

glass diameter of 10 mm which had an inner core of copper with a diameter of 8 mm. The plasma machine operated at a voltage between 220-240 volts with a frequency of 1.750 KHz. A helium gas flow rate of 1 L/min was used. Six mL of culture medium without FBS and antibiotic/antimycotics was added to a glass container of 40 mm diameter (50 mL beaker washed with deionized water 3 times and sterilized before use) which was placed on a stainless steel table due to its good electrical conductivity *(Fig. 2)*. NTP was generated 0.5 cm above the liquid surface and exposed for 60 sec. Three different treatment conditions were used; low dose (3.24 J/cm^2), medium dose (9.12 J/cm^2) and high dose (15.72 J/cm^2). The FBS and antibiotic/antimycotic were added after NTP treatment in order to prevent denaturation during exposure to plasma. NTP treated culture medium was then supplemented with FBS and antibiotic/antimycotics and is called the complete medium. The NTP treated complete medium was used to treat fibroblast cells immediately. The pH of cell culture was measured before and after exposure to plasma using a Consort C380 pH meter (Consort, Turnhout, Belgium).

Detection of Reactive Species in the Gas Phase

NTP species generated above the liquid cause a complex interaction with the culture medium and change its composition. Transmission of NTP components into the medium leads to the generation of secondary reactive radicals such as nitrate, nitrite and hydrogen peroxide [37-39,43]. The radical species in the NTP above the liquid surface and in the liquid medium were measured. Nitric oxide (NO) and Ozone (O_3) are stable molecules emitted by the gas NTP and result in generation of Nitrites (NO_2^-) and Nitrates (NO_3^-) in NTP treated medium. For the measurement of NO in the NTP, a gas detector (Shenzhen YuanTe Technology, Model SKY2000) was used with a precision of 1 ppb in the range of 0.05 to 100 ppm. For the measurement of O_3, a gas detector (Shenzhen YuanTe Technology model SKY2000) was used with a precision of 2 ppb ranging from 0.05 to 250 ppm. Each measurement was performed in triplicate.

Detection of Reactive Species in Culture Medium Treated With NTP

Comparison between three different NTP treatment doses: In the NTP treated culture medium nitrite (NO_2^-) is one of the reactive species products closely related to Nitric oxide (NO). Nitrite was used to indicate the presence of other

reactive species generated in the medium by the NTP. Nitrite was measured by the presence of the reddish-purple azo dye, formed by the coupling of diazotized sulfanilamide (diazonium salt) with N-(1-naphthyl)-ethylene-diamine dihydrochloride (NED dihydrochloride). The concentration range for this spectrophotometric measurement was 10 to 1.000 µg NO_2^--N/L. Higher concentrations of NO_2^- were determined by sample dilution [44]. Photometric measurements were made at 543 nm using a DU 730 UV-Vis Spectrophotometer (Beckman Coulter, Inc.; USA).

To determine nitrite generated in NTP treated cell culture medium, a standard curve of nitrite in culture medium without serum was created by plotting absorbance of standard against NO_2^--N concentration. Culture medium exposed to NTP was computed for the concentration of nitrite directly from the standard curve. The concentration of nitrite in NTP complete medium was measured to make a comparison between each NTP treatment instantly after treatment (0 h) and 30 min after treatment.

To compare the NO_2^- concentration created by 3 different NTP intensities, culture media without serum were exposed to a low dose (3.24 J/cm^2), a medium dose (9.12 J/cm^2) or a high dose (15.72 J/cm^2), each performed in triplicate. Colour reagent solution (240 µL) was added and well mixed. The solution was left for 30 min at room temperature before determining the absorbance.

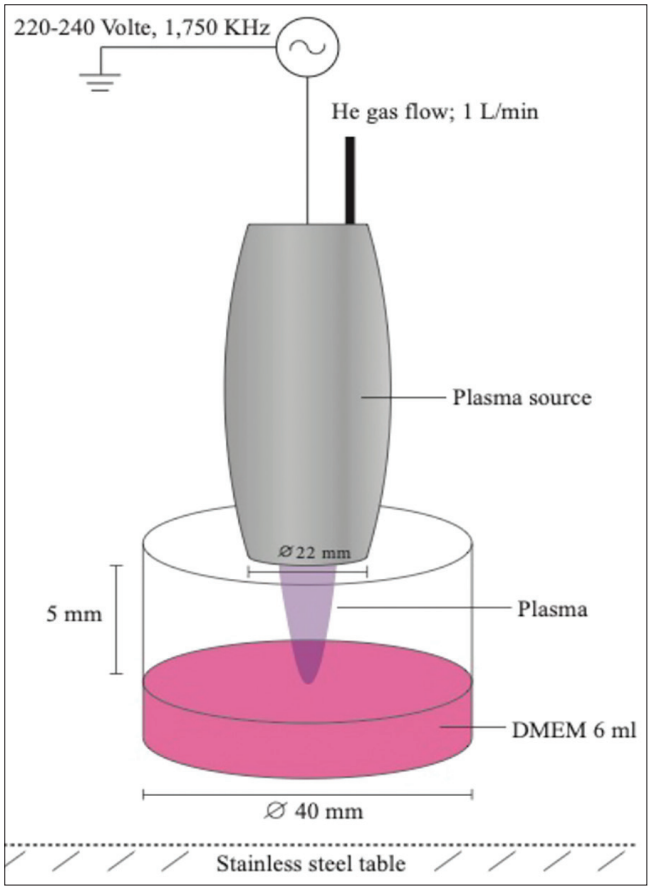


Fig 2. DBD helium jet exposed 6 mL culture medium without serum for 60 sec which contained in 50 ml beaker placed on stainless steel table

Monitoring NO_2^- in treated cell culture media for 24 h: To monitor NO_2^- created by NTP in cell culture medium for 24 h after exposure to NTP at low dose condition (3.24 J/cm^2), the concentration of NO_2^- was measured every 4 h in triplicate for 24 h. At 0 h condition, 240 µL colour reagent solution was added and mixed immediately after being exposed with NTP. The solution was left for 30 min at room temperature before determining the colour absorbance. For the samples at 4, 8, 12, 16, 20 and 24 h conditions, after being exposed to NTP, treated culture medium were left in incubator at 37°C, at 5% CO_2 condition, and measured for the absorbance with calculations to determine the concentration.

Determination of the Period of NTP Incubation Using MTT Assay

The viability of fibroblasts grown in cell medium treated with NTP was determined after different incubation periods. Fibroblasts were grown at a density of 10.000 cells/well in 96 well plates and cultured in complete medium with 10% FBS and 1% antibiotic/antimycotic 200 µL/well for 24 h at 37°C, 5% CO_2. The culture medium (before addition of FBS and antimicrobial addition) was treated with one of three NTP intensities: low dose (3.24 J/cm^2), medium dose (9.12 J/cm^2) and high dose (15.72 J/cm^2). Each medium was treated with NTP for a period of 60 sec. The NTP generating condition was the same as the condition for comparing NO_2 created by 3 different NTP intensities (see above). After seeding for 24 h the incubation medium was replaced with 200 µL NTP-treated complete medium and incubated for a further 24 h. After every 4 h the incubation medium was replaced with new complete medium. For measurement of viability cells were washed once with sterile PBS and then 200 µL MTT solution was added and incubation continued at 37°C, 5% CO_2 for 4 h (cells incubated in culture medium without MTT were used as blank controls). Dimethyl sulfoxide (DMSO) 100 µL was then added to each well and shaken for 5 min. The absorbance at 540 nm was then measured using a microplate reader. The viability of cells incubated in medium treated with NTP was expressed as a percentage of the viability of cells incubated in medium that had not been treated with NTP.

Measurement of the Total Cell Count, Viability and Proliferation of Elephant Skin Fibroblasts Incubated in Medium Treated With NTP

Fibroblasts were grown at a density of 100.000 cells/well in 6-well plates and cultured in completed medium for 24 h. Fibroblasts were incubated in medium that had been treated with zero, low, medium and high intensities of NTP. Consequently 2 mL of NTP-treated completed medium was replaced in each well, for each group of wells in triplicate. Similar to the control group, complete medium without

NTP treatment was continuously replaced and incubated at 37 C, 5% CO_2 condition for 24, 48 and 72 h with changed new complete medium 24 h after incubated with NTP-treated completed medium. Cells were investigated for total cells count, viability rate and proliferation rate using Muse™ cell Analyzer (Merck KGaA; Darmstadt, Germany) at 24, 48 and 72 h.

Total cell count and viability rate were measured by the Muse™ Count & Viability reagent (Merck KGaA; Darmstadt, Germany) which was fluorescent dye-based analysis. Dead and dying cells would lose their membrane integrity and allow the dye to stain the nucleus while live cells did not. This parameter was used to differentiate viable from non-viable cells.

For measuring the cell proliferation rate, the Muse® Ki67 proliferation assay (Merck KGaA; Darmstadt, Germany) was used to determine the percentage of cell's proliferation based on nuclear antigen ki67, which is expressed by proliferating cells in active cell cycle phase (G1, S, G2 and M phase) but absent in the resting phase (G0 phase). The Ki67 proliferation assay utilized ki67 expression to identify proliferating cells and distinguish them from non-proliferating cells or cells stained with IgG1.

Statistical Analysis

The results in this study are shown as a mean value ± standard deviation (SD) of data from three experiments. Measurement of the total cell count, viability and proliferation were performed as three experiments from the three different elephants and each experiment was replicated three times. Statistical significance was estimated by one-way analysis of variance followed by Dunnett T3 post hoc test. A P value <0.05 was considered statistically significant. All calculations were performed using the SPPS version 14.0 for Windows (SPSS, USA).

RESULTS

NO and O_3 Concentrations in the Gas Phase Generated by NTP Above the Liquid

Gas detector was placed at NTP emission point to measure the concentration of reactive radicals generated by NTP. As the NTP energy increased, the concentration of both reactive radicals, NO and O_3, also relatively increased *(Table 2)*. It was observed that using 100% Helium (He) gas flow rate 1 L/min, the concentration of NO was higher than O_3 at each of the three NTP intensities.

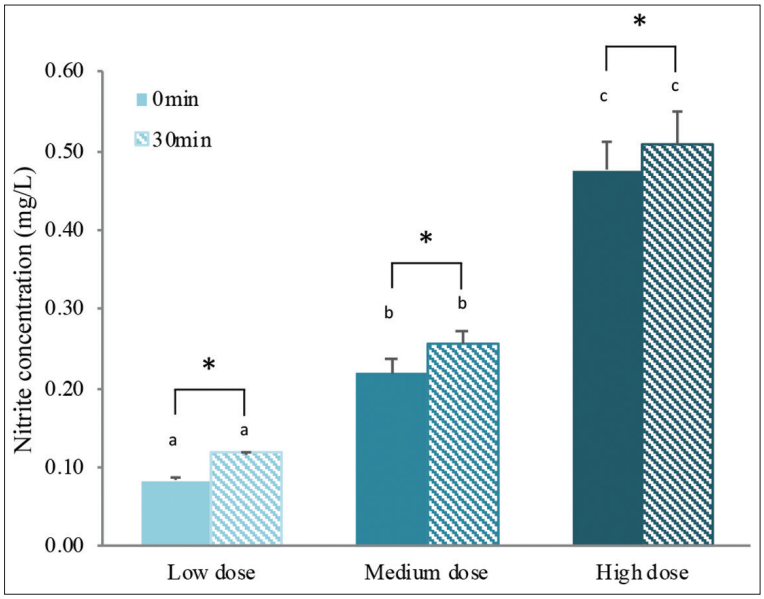


Fig 3. Comparison of nitrite concentration (mg/L) generated in culture medium between three different NTP treatment doses measured immediately and 30 min after exposure to NTP. Results are presented as mean ± standard deviations, each performed in triplicate. * show a significant difference (P<0.05) between measurements at 0 min and 30 min; ^{a,b,c} shows a significant difference between three NTP conditions at the same time

Nitrite (NO_2^-) Concentration in Culture Medium Treated With three NTP Intensities

The concentration of NO_2^- in media at 30 min after exposure with NTP was significantly higher than observed immediately after exposure (P<0.05) for all NTP intensities *(Fig. 3)*. The concentration of NO_2^- in media significantly increased with the NTP energy delivered *(Fig. 3)*.

Nitrite (NO_2^-) Concentration and pH in Culture Medium in the 24 h After Treatment With NTP

The concentration (mg/L) of NO_2^- detected in NTP-treated culture medium exposed to the low intensity condition (3.24 J/cm²) is shown in *Fig 4*. The concentration of NO_2^- 4 h after treatment was significantly higher than immediately after treatment (0 h) and thereafter remained approximately constant for 24 h. This ensured

Table 2. *Emission of NTP reactive radical volume in gas phase*

Parameters	Low Dose (3.24 J/cm²)	Medium Dose (9.12 J/cm²)	High Dose (15.72 J/cm²)
Nitric oxide (NO) (ppm)	3.32±0.13[a]	5.50±0.19[b]	7.48±0.23[c]
Ozone (O_3) (ppm)	0.43±0.6[a]	1.53±0.6[b]	2.67±0.32[c]

He gas rate 1 L/min, Relative humidity [45] 56%, Temperature 25°C. Superscript [a, b, c] show a significantly different (P<0.05) between treatments groups

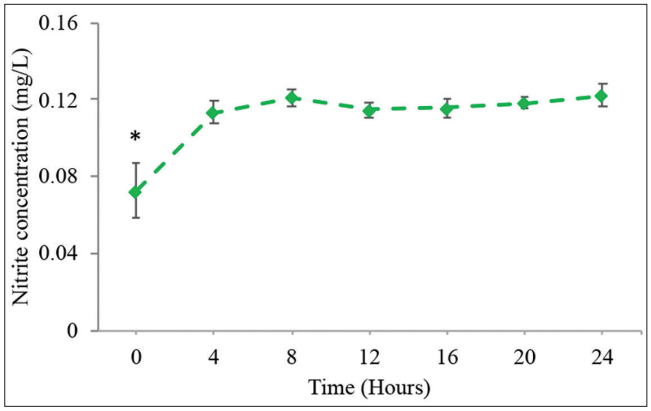


Fig 4. Measurement of the concentration (mg/L) of Nitrite generated in culture medium after being exposed with low intensity condition (3.24 J/cm2) for 60 sec. Results are presented as mean ± standard deviations, each performed in triplication. * show a significantly different (P<0.05)

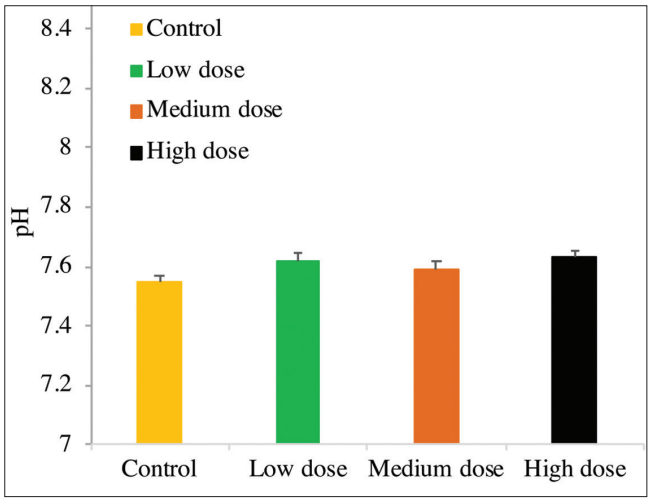


Fig 5. The pH of the cell culture medium after NTP treatment

that reactive species still remained for 24 in cultured medium for 24 h after treatment with NTP.

The pH of the cell culture medium was not influenced by treatment with low, medium or high NTP intensities *(Fig. 5)*.

Cell Viability at Different İncubation Times Measured Using the MTT Assay

The viability of fibroblasts was determined by MTT assay during 24 h of incubation in media treated with low, medium and high NTP intensities *(Fig. 6)*. The percentage of viable cells was higher than 90 percent, except for one time point (12 h) and one NTP intensity (medium).

Effect of NTP Treatment Conditions on Elephant Skin Fibroblasts

At the culture periods of 24, 48 and 72 h, the proliferation rate of fibroblasts in medium treated with NTP was significantly higher (P<0.05) than in cells grown in control medium (untreated with NTP) *(Table 3)*. The proliferation

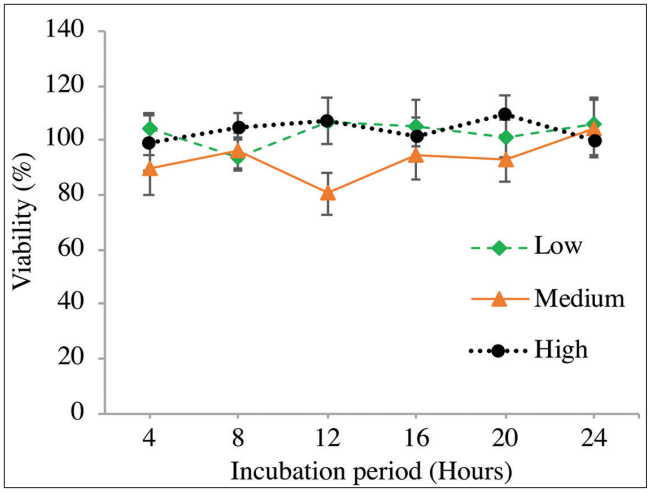


Fig 6. Viability of elephant skin fibroblasts after serial incubation with three different NTP treated complete medium conditions (low dose (3.24 J/cm²), medium dose (9.12 J/cm²) and high dose (15.72 J/cm²)); Cell viability (%) of fibroblasts was evaluated by MTT assay and data are presented as mean ± standard deviations, each performed in triplication

rate increased with increased intensity of NTP exposure from low to high *(Table 3)* (P<0.05). At 24 h, proliferation rate of the treated fibroblasts with low, medium and high dose were 2.54, 2.70 and 3.11 times higher than the untreated cells, respectively *(Table 4)*. At 72 h, the rate of the cell proliferation dropped which might be caused by the cells reaching confluence.

The number of cells in both the NTP treated and the untreated (control) groups approximately doubled in every 24 h *(Table 3)*. The average total cell count of all treatment groups was significantly higher than that of untreated group (P<0.05). The viability of cells at all time points was not significantly influenced by incubation in NTP treated medium *(Table 3)*.

DISCUSSION

Results from the current study has shown that the proliferation rate and total number of elephant skin fibroblasts grown *in vitro* was significantly higher (2-3 fold) when cultured in medium exposed to a NTP. Furthermore, proliferation rate and total cell count of treated fibroblasts was increased according to the NTP dose delivered. The study from Kalghatgi, Friedman [4] that used a low level (4 J/cm²) of NTP from dielectric barrier discharge (DBD) suggested that the reactive species generated interacted with cell membranes and caused sub-lethal cell membrane damage. The treatment of higher level NTP (8 J/cm³) lead to cell apoptosis and induced cell necrosis (or non-specific cell death) and inflammation. The different outcomes between our study and the findings of Kalghatgi, Friedman [4] may be caused by the use of different culture media [40,46] and NTP equipment.

The choice of culture medium may change the nature and

Table 3. *Effect of NTP treatment conditions on elephant skin fibroblasts*

Treatment Conditions		Cell Culture Period (h)		
		24 h	48 h	72 h
Proliferation rate (%)	Control	0.78±0.13[a],[i]	0.94±0.15[a],[ii]	0.84±0.11[a],[i,ii]
	Low dose	1.97±0.34[b],[i,ii]	2.05±0.27[b],[i]	1.74±0.25[b],[ii]
	Medium dose	2.10±0.28[b],[i]	2.45±0.32[c],[ii]	1.70±0.25[b],[iii]
	High dose	2.41±0.33[c],[i]	2.80±0.41[d],[ii]	1.92±0.24[b],[iii]
	Mean±SD	1.79±0.70	2.20±0.74	1.49±0.50
Total cells count ($x10^4$)	Control	238.362±19.479[a],[i]	411.209±5.973[a],[ii]	806.729±48.923[a],[iii]
	Low dose	252.632±12.731[a],[i]	473.822±44.505[b],[ii]	950.263±101.477[b],[iii]
	Medium dose	259.910±15.818[b],[i]	509.208±45,400[b],[ii]	1.037.017±76.506[b],[iii]
	High dose	285.991±24.234[c],[i]	512.399±37.057[b],[ii]	1.058.791±67.946 [c],[iii]
	Mean±SD	258.675±25.550	479.114±56.475	960.185±127.272
Viability rate (%)	Control	84.29±3.86	83.88±6.94	87.09±7.06
	Low dose	87.30±4.01	84.84±3.88	86.62±7.94
	Medium dose	87.63±6.45	87.57±6.88	90.57±5.50
	High dose	84.25±4.65	85.44±3.94	89.50±6.08
	Mean±SD	85.89±4.96	85.47±5.74	88.42±6.71

Superscript [a, b, c] show a significantly different (P<0.05) between treatments groups. Superscript [i, ii, iii] show a significantly different (P<0.05) between times within same treatment group

Table 4. *Proliferation rate related to control group*

Treatment (n=3)	Cell Culture Period (h)		
	24 h	48 h	72 h
Low dose	2.54	2.19	2.07
Medium dose	2.70	2.62	2.03
High dose	3.11	2.99	2.29

number of reactive species generated by NTP treatment. HEPES, a buffering agent for maintaining physiological pH of cell culture medium has a high ability to scavenge reactive species [41,46]. Furthermore, serum and antibiotics also have a minor radical scavenging ability [41,46]. In this study, fibroblasts that had been exposed with high dose NTP-treated complete medium with HEPES, serum and antibiotics showed the highest proliferation rate, compared to other treatment groups. Radical scavenger in culture medium was capable to scavenge reactive species which formed in culture medium after NTP treatment, and resulted in reducing oxidative effects inside the cells by decreasing of intracellular reactive species concentration [46]. This may the reason of using high dose of NTP treatment did not damage cells to death and reduced cell proliferation rate in this study.

NTP induced cell proliferation through intracellular reactive species formation [5], which bound to cell surface receptors and some can induce and generate new reactive species by interaction with cells components and consequently stimulated intracellular signalling pathways [4,41]. Increasing of intracellular reactive species also induced lipid peroxidation, changing of gene expression and cell membrane damage causing sub-lethal damage conditions which lead growth factors and cytokines release [4]. These intracellular mechanism resulted in a change of cell viability, proliferation, alter cell migration or induction of apoptosis which were dependent on NTP doses, treatment times together with cell types [41].

Among the consequences of generating NTP, radio-active emissions and UV-radiation potentially have the most effect on cells [41]. With the exposure of cells to NTP-treated culture media, the direct effects of UV-radiation can be excluded [47]. While direct treatment with NTP would allow cells to be exposed with all NTP components [41], some NTP components will be transferred into the treated liquid at the area of fluid exposed with NTP and will lead to reactive species production in the bulk liquid or liquid-gas interface [38,47]. NO, reactive nitrogen species, and O_3, reactive oxygen species, are one of the main radical species generated by NTP [48]. The amount and proportion of various reactive radicals generated in gas phase depend on plasma gas source [49,50]. In this study which uses Helium plasma, the ratio of NO radical emitted was higher than O_3. As with other studies, it was found that oxygen plasma generated a large number of singlet oxygen and OH radical. Whereas for air plasma, OH radical was less produced. However, the concentration of nitrate and nitrite in the liquid is higher than that produced by other gas plasma. This might be because air plasma generated a large number of NO and NO_2 [49]. Moreover, this study showed that concentrations of NO and O_3 were increased in the gas above by NTP in a dose-dependent manner. In

the study of Kim et al.[48], increasing of plasma treatment time also affects the increase in the amount of oxygen radicals.

After NTP generated plasma species above liquid surface, radical species were delivered as gas phase and then transferred, or some formed secondary generation of reactive radicals at the plasma-liquid interface, leading to chemical changes in the exposed liquid. For instance, nitrate and nitrite are the result of the reaction of plasma generated RNS such as NO and NO_2 and liquid [49]. This study showed that nitrite concentration (mg/L) generated in culture medium increased according to the energy dose delivered and remained in the NTP-treated complete medium for an extended period of time (up to 24 h in this study). This is because these generated radicals are long-lived, so species that can cause these reactions can spread to liquid without losing [47]. However, exposure to plasma with different energy will cause different chemical changes in the exposed liquid, depending on the characteristics and composition of the liquid [40,47].

Plasma radicals which dissolved in treated liquid were the cause of changing of pH by reacting with solution and also generating of secondary relative radicals. However, being buffer solution of culture medium resulted in almost no change of pH after plasma treatment. Consistent with the study of Trizio et al.[40], after plasma treatment, pH of distilled water was altered while culture medium and PBS did not noticeably change. Differing from other fluids e.g. water and PBS, culture medium solution containing amino acids, protein, serum and others organic components interact with reactive species generated by NTP and produced long-lived reactive species in treated culture medium [51]. In addition, there are other factors that influence the amount of ROS generated; the type of NTP machine, the NTP type, the gas generator, the gas flow volume and the materials of the table, and other environmental factors such as temperature and humidity.

Although this study successfully showed that medium treated with NTP was able to stimulate elephant skin fibroblasts *in vitro*, there were some limitations of the study. A monolayer cell culture does not resemble the wound on the skin of an elephant. There are many factors involved in the healing process, such as the interaction of various cell types, cytokines, wound conditions and overall animal health, which might affect the performance of NTP liquid treatment. Further studies using NTP treated liquids on elephant skin wounds are encouraged to develop the most effective and optimum treatment conditions.

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