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Inactivation of *Eimeria* oocysts in aqueous solution by a dielectric barrier discharge plasma in contact with liquid

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Keywords: cold atmospheric-pressure plasma, dielectric barrier discharge, biological effects, biocidal efficacy, parasites, coccidia

Abstract

This study presents a novel technique to inactivate coccidian oocysts in an aqueous solution. The technique consists in treating the contaminated liquid by using an atmospheric-pressure air Dielectric Barrier Discharge (DBD) plasma in contact with it. Many experiments in several operating conditions were performed. The discharge was supplied by sinusoidal and nanosecondpulsed voltages with a constant average power of ~ 7 W in both cases. Biological effects due to the plasma were investigated by performing tests with increasing treatment time. A sudden ~ 40 % drop in the number of survived oocysts was reached in 4 min and a two-fold reduction was detected after 12 min of exposure. No significant differences in the biocidal efficacy were detected between the AC-driven and the nanosecond-pulsed discharge. Chi-squared statistical analysis on

the treated samples showed significant statistical difference (with a statistical significance P-value parameter smaller than 0.01) and non-randomness warranty of the results, opening interesting scenarios for future developments.

Key words: cold atmospheric-pressure plasma, dielectric barrier discharge, biological effects, biocidal efficacy, parasites, coccidia.

1. Introduction

Amongst human and animal diseases agents, parasites show much higher environmental resistance than the others [1]. Their complex wall structure in the infective stage makes parasites resistant to almost all physical conditions and chemical compounds [2]. An example of parasitic element are coccidia, whose oocysts may last more than 600 days in soil [3]. Moreover, *Toxoplasma gondii* and *Cryptosporidium parvum*, two of the most severe zoonotic agents worldwide [4], belong to the same order (Eucoccidiorida) and suborder (Eimeriorina). Coccidia are intracellular obligated protozoa, particularly feared in poultry and rabbits breeding. They have been responsible for serious economic losses since the first half of the 20^{th} century [5]. Breeders have tried to control coccidiosis over the years by means of feed water added with

pharmaceuticals, thereby increasing the risk of drug resistance [6].

Cold atmospheric-pressure plasmas have been widely investigated in the past two decades for biocidal purposes [7, 8] and they have been applied with great efficacy to kill several pathogenic microorganisms thus far [9, 10, 11, 12, 13, 14, 15, 16, 17]. One of the most attractive features of cold atmospheric-pressure plasmas is their capability to enhance chemical processes without the need for high temperatures. They have been established to be energy-efficient and eco-friendly novel tools for the simultaneous production of intense UV radiation and extremely strong oxidising species (O_3, $OH\cdot$, H_2O_2) [18], which are all effective against pathogens. Therefore, the versatility and low operational cost of room-temperature plasmas offer the possibility for interesting collaborations.

In this work, a novel method to inactivate coccidian oocysts is successfully explored. This technique involves the treatment of an aqueous solution containing the parasites by means of an atmospheric-pressure air Dielectric Barrier Discharge (DBD) non-thermal plasma in contact with it. The discharge was supplied by both sinusoidal and nanosecond-pulsed voltage waveforms, keeping constant the average power. Several experiments with increasing treatment time were carried out. The lack of vacuum facilities, the usage of atmospheric pressure air and the very limited temperature increments, allow to use of this apparatus directly into a 'real life' scenario.

2. Materials and methods

The coccidia *Eimeria necatrix*, *E. maxima* and *E. acervulina* were collected in poultry farms with clinical coccidiosis evidences. These coccidian types have been selected because of two main reasons. The former is related with the presence of an intermediate environmental stage where these coccidia should be very resistant and where we would like to intervene. The second reason is related

with their relatively large size, of about 50÷100 microns. This allows us to morphologically evaluate the infectivity of oocysts without the need of experimental animals and avoiding the risk of infections for technicians.

The oocysts were not extracted directly from the animal intestine, but they were taken from litters to ensure complete sporulation. The litters were washed with spring water, sieved and sedimented in conic goblets with a volume of 1 L to increase the parasites concentration. The coccidian were gently washed every 30 min until a clear supernatant fluid was obtained. The tubes containing the suspension were overturned several times and then 20 mL of contaminated liquid was poured into a polystyrene Petri dish with diameter of 90 mm to perform plasma treatments.

The Petri dish was placed between the electrodes of a plane-to-plane DBD device (figure 1). The high voltage electrode was a stainless-steel plate with diameter of 100mm. A 3mm thick ceramic slab with surface area of 50×50 mm^2 was used as dielectric substrate. A 30×30 mm^2 copper strip was glued onto the insulating layer and connected to the ground.

The electrodes were supplied by both sinusoidal and nanosecond-pulsed waveform voltages to ignite an atmospheric-pressure air DBD plasma above the contaminated liquid surface and in contact with it (figure 2). The amplitude of the sinusoidal voltage was set at 18 kV at a frequency of 4.7 kHz. When the nanosecond-pulsed power supply was used, peak voltage and pulse repetition frequency were set at 10.2 kV and 1 kHz respectively. The average power delivered to the discharge was ~ 7 W in both cases. The air gap was set at 3 mm in the case of AC voltage
(figure 2a), whereas it was adjusted at 1 mm in the case of nanosecond-pulsed excitation (figure

2b). The plasma presented filamentary or homogeneous behaviour accordingly to the excitation voltage waveform, thereby leading to different local properties and chemical processes even though the average power was kept constant. The DBD device used in this study has been already characterised in [19]. Refer to that article for in-depth details on the operating conditions, discharge physics and chemistry.

The contaminated liquid was exposed to the plasma for 4, 8 and 12 minutes and one control sample was prepared for each of the two considered excitation voltage waveforms. The treated suspensions were diluted with an equal volume of a 2.5% potassium dichromate solution ($K_2Cr_2O_7$) and then transferred in labelled sporulation chambers to obtain the infectivity stage (figure 3). The content of each sporulation chamber was centrifuged for 5 min at 1500 rpm seven days after the plasma treatment and suspended again in flotation solution with specific gravity 1.3. A small amount of liquid at the top of the tube was then analysed by means of an optical microscope. Hundred coccidia were isolated for each specimen and the number of survived (sporulated) oocysts after the plasma treatment was counted. The number of unsporulated and damaged was monitored as well (figure 4). Unsporulated oocysts (a) consist of a nucleated mass of protoplasm enclosed by a resistant wall, sporulated oocysts (h) consist of an outer wall enclosing four sporocysts each one containing two sporozoites (infective stage) and damaged oocytes (c) present a clear wall post-treatment damage. The damaged oocysts are non-infectious and, therefore, were not considered in the survived count. The results were evaluated via chisquared test using the software EpiInfo [20].

3. Results and discussion

Treatments were performed in triplicate and the standard deviation between experiments was < 7%. Results can be summarised as follows.

Although the DBD device generates very different plasmas accordingly to the supply voltage waveform, the number of survived and non-infectiuos oocysts after the treatment does not seem to depend strongly on the discharge excitation waveform. The graphs (figure 5) show comparable trends and data are within error bars in all the cases. Conversely, the plasma exposure time seems to be playing the major role. The number of survived parasites (figure 5a) presents a ~40% drop in the first 4min and a ~two-fold reduction is reached after 12min of experiment. The number of non-infectious oocysts (figure 5b) quite mirrors this behaviour with an increasing trend over the time, though.

These results suggest that the coccidian inactivation mechanism is dominated by the damaging action of the discharge (table 1). The production of reactive species in liquid induced by the plasma are responsible for the parasite higher damage rate as the treatment time is increased. The DBD device used to perform the treatments has been already proven to be efficient in generating ozone, hydrogen peroxide and acids in the treated liquid without significantly increasing its temperature [19]. All these chemical compounds oxidise the oocysts wall structure, thereby leading to their inactivation. However, even if the nanosecond-pulsed discharge has been already demonstrated to

be much more effective in enhancing the liquid-phase chemistry [19], the sinusoidal plasma shows a slightly higher inactivation efficiency, especially for longer test times.

This result is still not completely clear, but may be justified considering the AC-driven discharge streamer-like behaviour. As mentioned above, the sinusoidal excitation leads to a filamentary plasma in which many streamers appear randomly in the discharge gap as soon as the breakdown voltage is reached. Those filaments propagate in the discharge gap at about 100 km/s [21] and, therefore, may damage the oocysts wall by the impact of the particle accelerated in the discharge as well as for the chemical processes they induce in the liquid.

The statistical analysis performed with the chi-squared test for both treated samples (different times and DBD sources) and control ones, presented a significant statistical difference a statistical significance P-value parameter < 0.01, thereby demonstrating the plasma effectiveness to reduce coccidian infectivity (table 2). These results show the chances to avoid two treatment times (8 min, 12 min). Despite the negative trend of the survival curve (figure 5a), this is not supported by statistical analysis.

4. Conclusions

This work shows a novel technique to inactivate coccidian parasites suspended in aqueous solution. The method here presented consists in exposing the contaminated liquid to an atmospheric-pressure

DBD non-thermal plasma in contact with it. Several experiments with different operating conditions were carried out. The plasma was fired by means of both sinusoidal and nanosecond-pulsed input voltage with a water temperature increment within few degrees. The average power delivered to the discharge was kept constant in both cases and several experiments with increasing treatment time were performed.

The plasma treatments provided a ~ 50% drop on the number of survived oocysts after 12min of exposure. No significant differences in the usage of the two different excitation sources was detected. Chi-squared tests on the plasma-treated coccidian compared with control, pointed out the stochastic warranty of non-randomness efficacy. This means that if the essay is repeated 100 times it should lead to the same results for 99 times. This medical statistical approach will allow us to reduce the number of the treated samples in future tests.

The inactivation results here presented are encouraging, but some aspects related to the coccidian inactivation mechanism are still unclear and need more investigations. To conclude, this method could pave the way for potential applications in the fields of medicine, veterinary medicine and public health. Further work is needed to optimize the treatments and develop more efficient plasma devices, though.

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Tables

	Sinusoidal			**Nanopulsed**	
	S	**NI**		**S**	**NI**
K	50	50	**K**	50	50
4'	30	70	**4'**	30	70
8'	27	73	**8'**	29	71
12'	25	75	**12'**	28	72

Tab.1: Results of two different test. S: sporulated; NI: non-infectious (unsporulated+damaged), K: control.

Sinusoidal		**Nanopulsed**	
K vs 4'	χ^2=7.52 P=0.006	**K vs 4'**	χ^2=7.52 P=0.006
K vs 8'	χ^2=10.22 P=0.001	**K vs 8'**	χ^2=8.37 P=0.003
K vs 12'	χ^2=12.29 P<0.001	**K vs 12'**	χ^2=9.27 P=0.002

Tab.2: Statistical differences between control (K) and treated samples ($P < 0.01$) under the two different DBD sources.

Figures captions

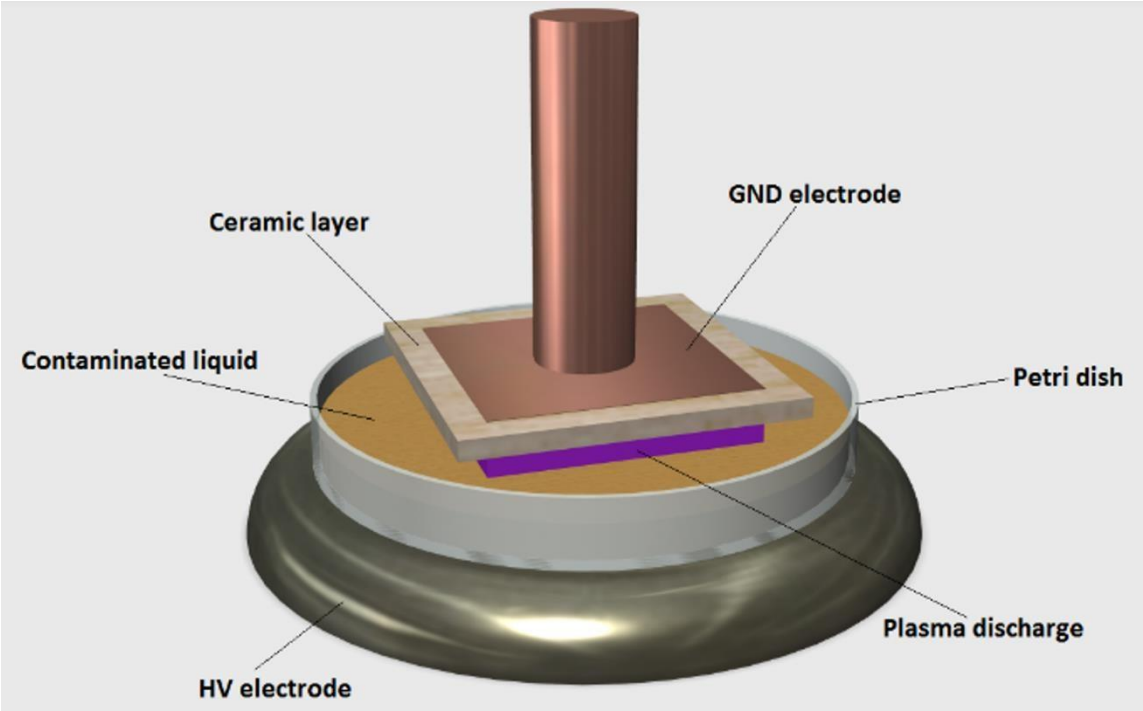

Fig.1: Schematic of the DBD device.

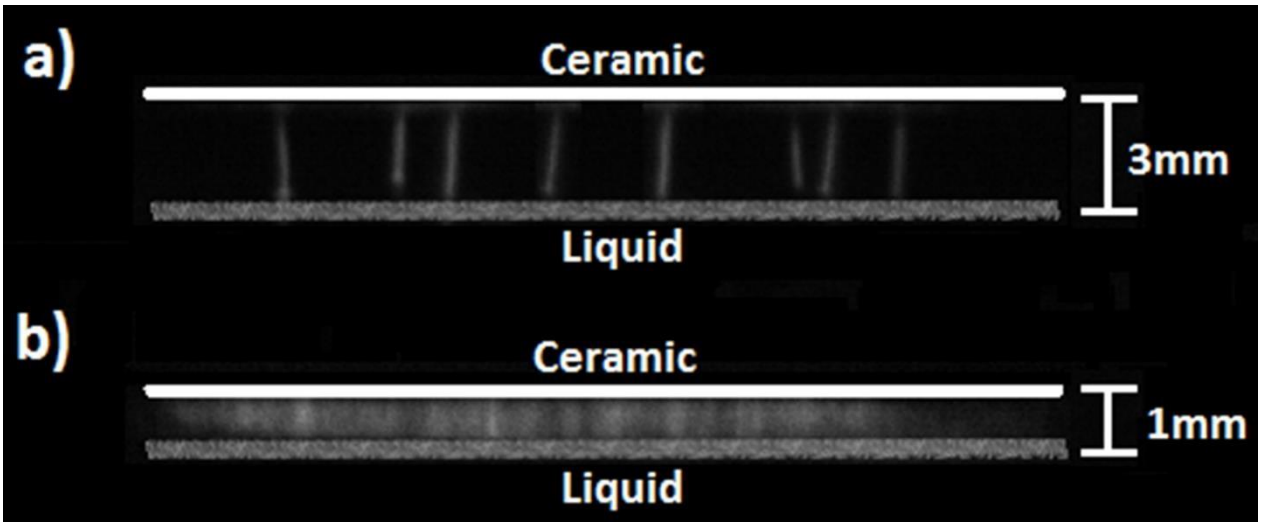

Fig.2: iCCD pictures of the sinusoidal (a) and nanosecond-pulsed (b) DBD plasma. Exposure time 210 µs (a) and 1 µs (b).

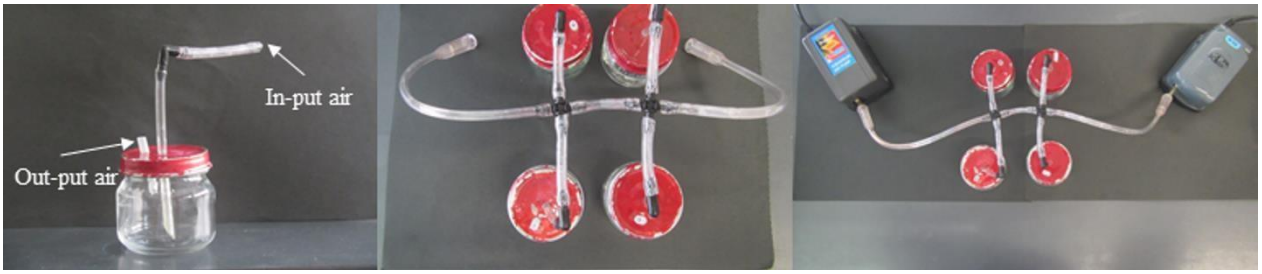

Fig.3: The sporulation chambers were obtained by means of glass jars, in which continuous oxygenation of the biological samples was guaranteed by means of two aquarium pumps.

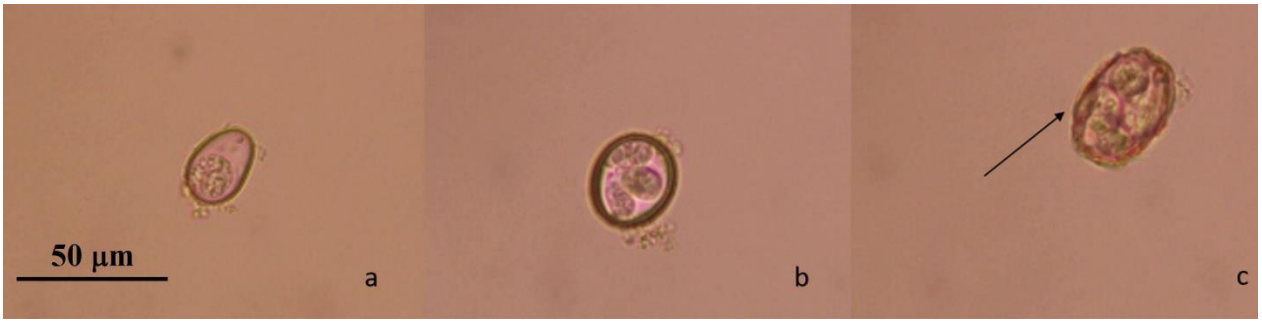

Fig.4: Images obtained by using the optical microscope. The picture shows a) unsporulated oocyst, consisting of a nucleated mass of protoplasm enclosed by a resistant wall; b) sporulated oocyst, consisting of an outer wall enclosing four sporocysts each containing two sporozoites (infective stage) and c) oocyst's wall post-treatment damage.

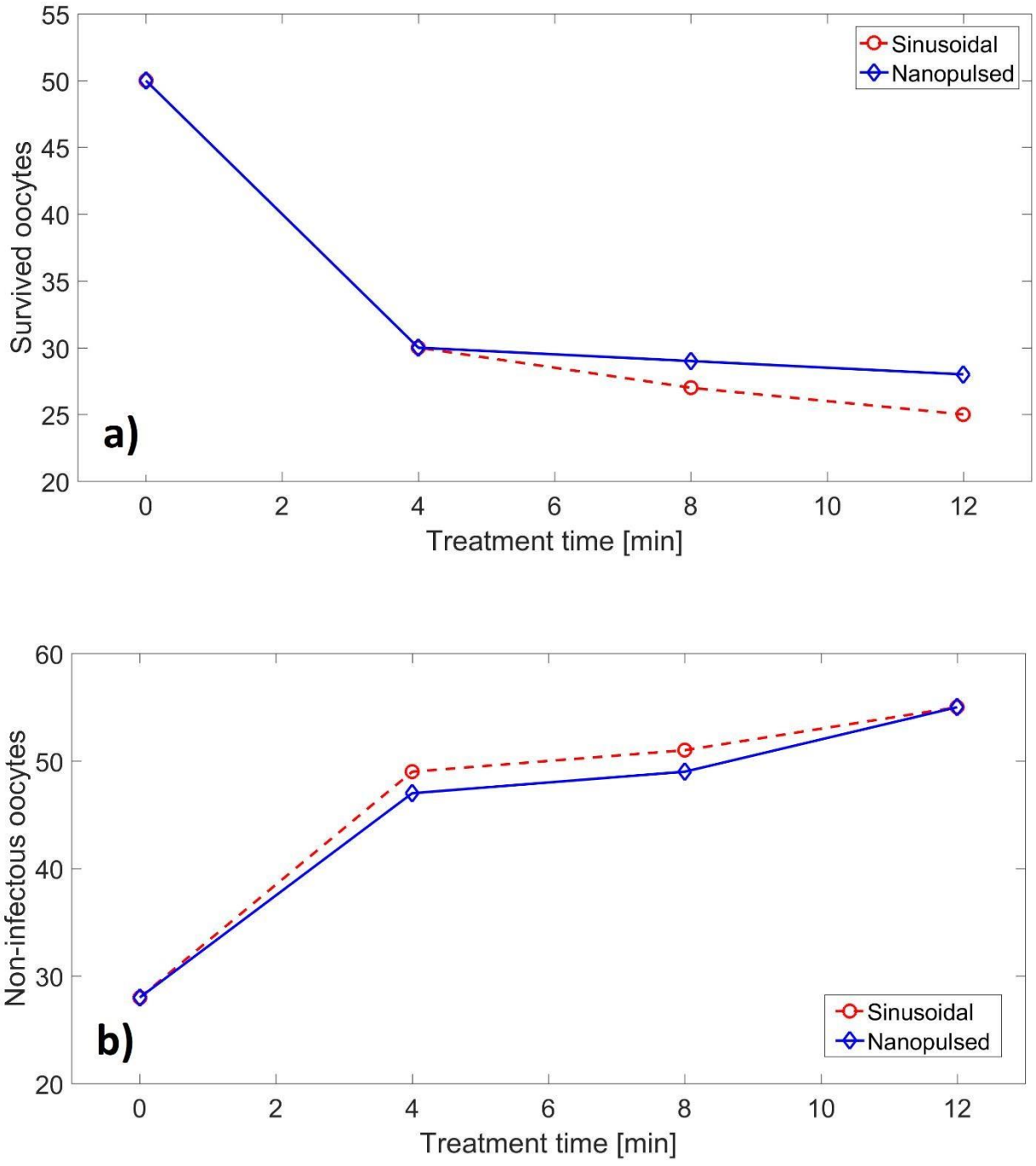

Fig.5: Number of survived (a) and non-infectioous (b) oocysts against treatment time.