

Cold Spark Discharge Plasma Treatment of Inflammatory Bowel Disease in an Animal Model of Ulcerative Colitis

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ABSTRACT: Cold plasma discharges have been shown to have medically-relevant therapeutic effects when applied to living tissues, including blood coagulation and wound healing; thus, plasma treatment of inflamed tissues in ulcerative colitis disease may be an effective approach to reduce adverse consequences if not cure the disease. Here we discuss results of the first experimental study of cold plasma treatment of ulcerative colitis in a mouse model. As a plasma source, a cold spark discharge has been used. The results show that plasma treatment of experimental model of ulcerative colitis in mice has some beneficial effects by suppressing the progression of the disease while no damage to colon tissues is observed; and these effects are comparable to standard therapy.

KEY WORDS: non-equilibrium plasma, cold spark discharge, animal model, plasma therapy, plasma medicine

I. INTRODUCTION

Recently, non-thermal atmospheric pressure plasmas have emerged as a promising new tool in medicine. Compared to conventional thermal plasma¹⁻⁵, cold plasma is selective in its treatment while no macroscopic tissue damage is observed. Non-thermal plasmas generate free radicals, charged species, and ultraviolet radiation, which may be used for targeted chemical modification and catalysis^{6,7} unlike thermal plasmas which employ high temperature that causes significant thermal tissue desiccation, burning, and scar formation. An example of thermal plasma is the Argon Beam or Argon Plasma Coagulator (APC) developed mainly to cauterize wounds⁸. On the other hand, cold plasma discharges have many potential medical applications that include sterilization of living tissue without macroscopic damage⁹, blood coagulation¹⁰, induction of apoptosis in cancer and other cells^{11,12}, and control of cell attachment¹³. Another promising field of cold plasma applications in medicine is gastroenterology, where strong antibacterial effect can be successfully combined with healing and anti-inflammatory effects. Thus, the focus of this study is to evaluate whether cold plasma in vivo is detrimental to gastro-

intestinal tissue in health and disease conditions and whether cold plasma provides any therapeutic benefit in an animal model of murine experimental colitis. The disease in this model produces experimental colitis reminiscent of human ulcerative colitis, which is a form of inflammatory bowel diseases. Inflammatory bowel diseases (IBD) consist of two major chronic, relapsing and debilitating forms of diseases known as ulcerative colitis and Crohn's disease that affect the gastrointestinal tract. The etiology of these diseases remains a mystery though genetic, environmental and immunological factors are found to play a major role in the induction, chronicity and relapses of these diseases. Crohn's disease may appear in any part of the gastrointestinal tract from the mouth to anus and affects the entire thickness of the bowel wall. On the contrary, ulcerative colitis is an inflammatory disorder affecting colonic mucosa and sub-mucosa¹⁴. There are no known curative therapies for these diseases; however, recent advances in IBD therapeutics have shown that certain biological therapies have been successful in maintaining remission particularly in Crohn's disease^{15,16}.

This study was designed to evaluate the effects of cold plasma treatment of both healthy colon tissue and experimentally-induced ulcerative colitis disease in a live animal model. The goals of the study were to examine whether cold plasma treatment adversely affects the mucosa in normal condition, and to evaluate if cold plasma treatment results in acceleration or worsening of the disease during its induction phase. Additional pilot experiments were conducted to study whether cold plasma discharges provide therapeutic effects, and whether these effects are comparable to a standard therapy, or cold plasma treatment enhances the beneficial effect of a standard therapy.

II. MATERIALS AND METHODS

A. Cold Pin-to-Hole Spark Discharge (PHD) Plasma

Currently, the primary reason of gastroenterological inflammatory diseases, and particularly ulcerative colitis, is still unknown; however, two major candidates are bacterial infections and autoimmune disorders. In order to be able to address these problems using plasma, the treatment should meet several conditions, namely:

- Average plasma temperature should be low, i.e. close to room temperature, when there is no or minimal thermal damage due to contact of plasma with tissues;
- Have strong bactericidal effect;
- Be able to provide anti-inflammatory effect.

Another set of criteria which are important in the present mouse model experiments refer to plasma system engineering problems:

- Electrode system ("plasma probe") should be small (maximum outer diameter is approximately 2 mm) in order to cause minimal mechanical damage to colon tissue of a mouse;

- Plasma should be easily ignited inside of a colon, and should not be sensitive to the distance between electrode and inner colon wall.

All these conditions may be satisfied if one is using cold spark discharge plasma. Dr. Gostev and colleagues reported that this cold discharge^{17,18} (authors used similar system as in the current study, but of about 10 times greater in size) is extremely effective in sterilization of bacteria both in liquid¹⁹ and on tissue surface²⁰, and due to production of significant amount of nitric oxide (NO) has pronounced “healing” effect through faster tissue regeneration, and other beneficial effects^{17,18,21,22}. The pin-to-hole electrode configuration makes the discharge ignition process not sensitive to the presence of surrounding tissues and natural liquids, in contrast to direct methods of creating arc, dielectric barrier, or corona discharges where treated surface is used as a second electrode. Also, PHD treatment allows operator to provide relatively high energy input resulting in shorter treatment time and less discomfort to an animal.

In our study we have used modified PHD system which consists of central copper needle covered by dielectric material which is inserted into a grounded stainless steel cylindrical electrode (Figure 1). In order to cause minimal mechanical damage to colon tissues external electrode is covered by polyethylene sleeve. The discharge was ignited by applying high positive potential to the central electrode. In order to provide high discharge energy while keeping average gas temperature low, the electrode system was powered through a capacitor. This resulted in a formation of dense energetic spark which exists for about 3.5 μs . Due to low repetition frequency of about 7 Hz and short pulse duration, the average gas temperature did not exceed room temperature.

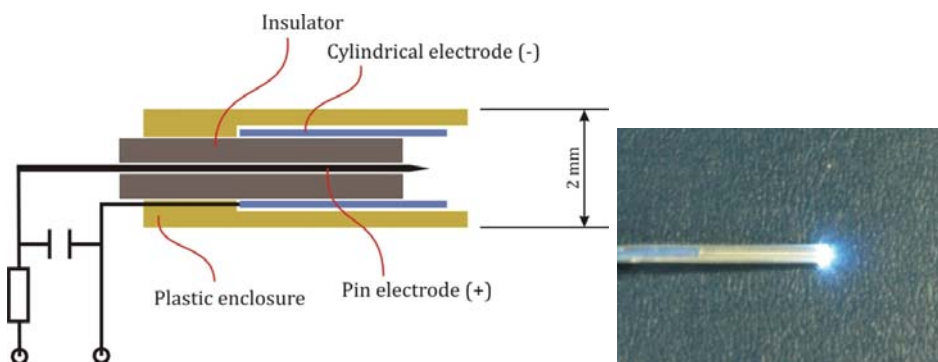


FIGURE 1 General schematic of the Pin-to-Hole spark Discharge (PHD) plasma system, and photograph of the discharge in operation.

The main average PHD plasma characteristics are the following:

- Peak voltage: 3.2 kV;
- Pulse duration: 3.5 μ s;
- Frequency: 7 Hz;
- Energy per pulse: 0.1 J;
- Plasma diameter: \sim 2 mm;
- Typical waveforms are shown in Figure 2.

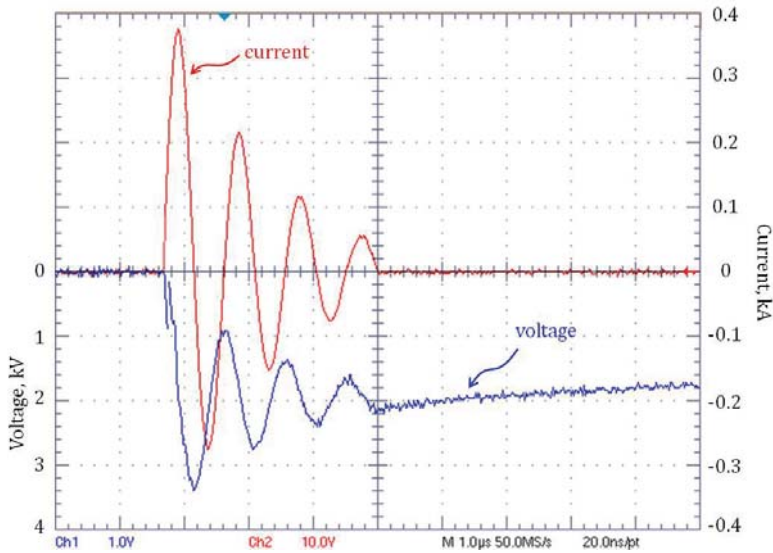


FIGURE 2 Typical voltage and current waveforms of cold Pin-to-Hole spark Discharge (PHD) plasma.

Although the average gas temperature is low, plasma temperature itself in order to cause production of significant amount of NO is expected to be relatively high. We have estimated plasma temperature using Boltzmann plot method^{23,24} which may be applied for estimation of gas temperature of sparks²⁵. This method uses the relative ratio of emission intensities on multiple spectra lines. Under plasma conditions at local thermal equilibrium, the plasma temperature is derived from the following equation:

$$\ln \frac{I\lambda}{Ag_u} = -\frac{1}{T}E_u + \ln C,$$

where I is the relative ratio of the emission intensity, λ - the wavelength, A - the transition probability, g_u - the statistical weight of the upper level, E_u - the energy of the upper level, C - a constant for various atoms. We used spectral lines of copper (material of central electrode) to estimate the temperature in the center of the discharge. The transition probabilities, the statistical weights of the upper level and the energy of the upper

levels of the copper lines are shown in Table 1²⁶. The copper emission lines from the spark discharge were measured using Acton SpectraPro 500i spectrophotometer; Figure 3 shows the result obtained which indicates that plasma temperature of about 7200 K, which is sufficient to generate significant amount of NO.

TABLE 1. Parameters of copper spectral lines³⁹

Wavelength λ , nm	Transition probability $A \times 10^8 \text{ s}^{-1}$	Statistical weight, g_u	Upper energy level E_u , eV
510.55	0.02	4	3.822
515.32	0.60	4	6.195
521.82	0.75	6	6.196

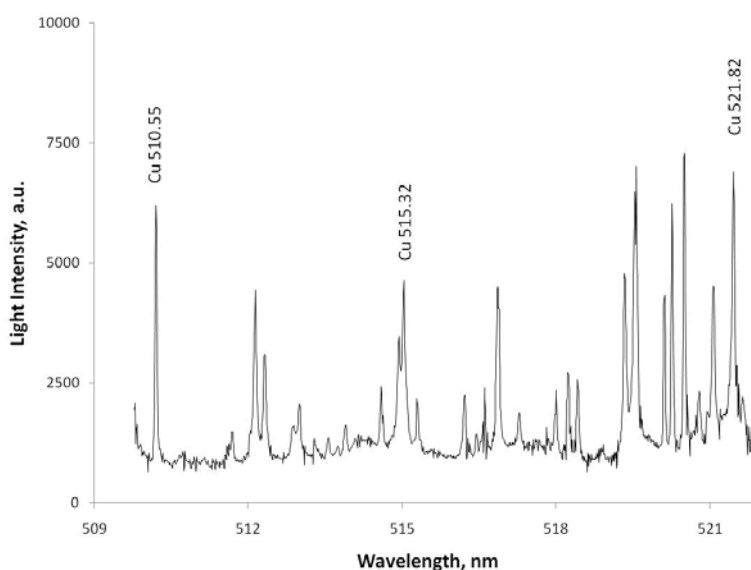


FIGURE 3 Spectrum obtained from spark discharge plasma in room air with the copper spectral lines used for plasma temperature estimation identified.

As can be seen from spectrum of radiation (Figure 4), PHD plasma radiates intensely in the UV range. In order to be sure that plasma will cause minimal DNA damage to cells due to direct exposure to UV, the outer tubing was made of about the size of plasma diameter, i.e. about 1.5 mm, longer than electrode system, covering the discharge from the sides. In addition to that we have measured total amount of UV irradiated by plasma using IL1700 photometer (International Light Technologies) to be about $5 \pm 1 \mu\text{W}/\text{cm}^2$. Recent studies show that DNA damage induced by UV-C and UV-B radiation occurs after about $0.4 \text{ mJ}/\text{cm}^2$ and $10 \text{ mJ}/\text{cm}^2$ respectively²⁷. Considering the fact that most of

UV radiation of PHD plasma comes from UV-C band, minimum 60 seconds of plasma treatment should be safe, causing none or minimal and reversible DNA damage to colon tissue.

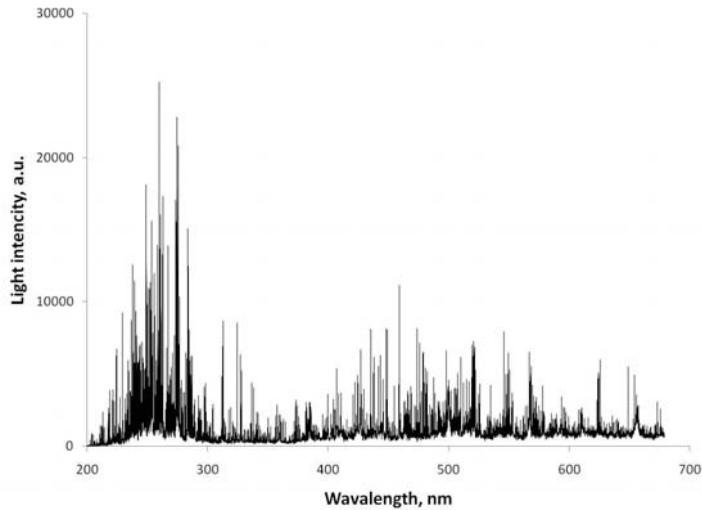


FIGURE 4 PHD plasma spectrum in room air

Lastly, it was necessary to check if miniaturized PHD plasma system indeed produces significant amount of NO, thus delivering the therapeutic effect (so-called, “NO-therapy”²⁸⁻³¹). The NO production was measured using gas chromatograph Agilent 3000 MicroGC, calibrated with 700 ppm NO balanced with nitrogen. The discharge cell was inserted inside of a syringe tip, and plasma treated room air was collected with slow plunger pulling during plasma treatment. Then, collected air was analyzed chromatographically. The results of our measurements (see Figure 5) show that NO concentration is varied from 900 to 1200 ppm depending on plasma treatment dose, which allow us to expect that plasma treatment of colon ulcer may cause “healing” effect.

Although one can argue that plasma characterizations mentioned above were made in different atmosphere (room air), and therefore the results could differ from real experimental situation when plasma is ignited inside of intestine, we do not expect qualitative difference between these two conditions. This assumption is based on data on gas composition inside of a colon, which is mostly consists of air-like mixture (up to 80% of N₂ and 2.3% of O₂) with small additives of hydrogen, methane, and carbon dioxide³². Moreover, before the treatment procedure, the colon was cleansed with saline, and also room air was introduced into the colon during probe insertion. Therefore, air is expected to be a dominant plasma-forming gas.

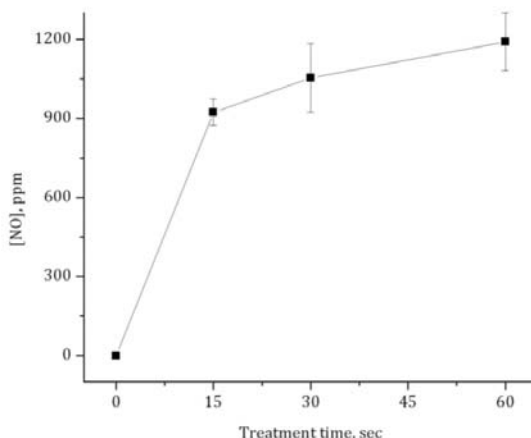


FIGURE 5 Nitric oxide production by PHD plasma

B. Dextran Sodium Sulphate (DSS) Induced Experimental Colitis in Mice

The DSS model was used in the current study to produce ulcerative colitis in mice which is representative of human ulcerative colitis^{33,34}. The disease is induced in an animal through a daily oral administration of 2.5% DSS dissolved in drinking water at concentration of 2.5%. Animal develops an acute form of inflammation beginning on the third day and they have a full blown colitis on the day of DSS feeding cycle. The primary characteristics of acute inflammation are the increased number of neutrophils in the mucosal layer, shortening of the epithelial crypts, hyalination in the lamina propria, accompanied by severe weight loss, diarrhea, and blood in the stool. This form of the disease gives an opportunity to use it as a model in efficacy studies of drugs and compounds³⁴. The most striking feature of this model is that it works using a very simple pathway to produce the disease as DSS overcomes the barrier of the epithelium to expose the mucosa to the flora present in the intestine resulting in an inflammatory response; this in turn leads to activation of macrophages and monocytes. The model also shows close links to the disease in human beings and it is simple to induce and reproduce³³⁻³⁵.

To quantify the disease induced by the DSS model a Disease Activity Index (DAI) have been used³³. A scale of 0 to 4 is used to quantify the disease with 4 being the lethal stage of the disease. DAI is scored on the parameters of weight loss, consistency of the stool and presence of blood in the stool. This index has been shown to be in a linear correlation with the histology score based on changes in the architecture of the crypt³³.

C. Animal Study Design

All experiments were done on live animals. The study consisted of 3 stages:

1. Toxicity study of plasma treatment of a colon.

2. Study DSS model disease progression after plasma treatment.
3. Comparison of effectiveness of plasma treatment with conservative therapy

In all the experiments Female Swiss Webster mice approximately aged six to eight weeks of 25 to 30 grams in weight were used. Prior to plasma treatment animals were anesthetized with 0.4 ml Nembutal®. The effect of the anesthetic was checked using the toe relax by pinching. The colon was cleansed with saline before inserting the probe.

III. RESULTS AND DISCUSSION

A. Toxicity of Plasma Treatment of Colon Tissue

The primary objective of the study was to ascertain if cold plasma treatment would cause any damage, to colon tissue or to the animal itself. To ascertain this effect, 12 mice were divided into 4 groups of three animals in each group. The first group was a control group which did not receive any treatment and the other three groups remained as experimental groups. In experimental groups a laparotomy was performed and the colon was exposed and kept moist covered with a saline gauze. The plasma probe was introduced through the anal verge up to 4 cm into the colon (Figure 6). Plasma treatment was administered for 0, 4, 30 and 60 seconds in the respective group (for “0” time point, the probe was inserted into the colon with no plasma ignited). To check colon tissue damage, mice were intravenously injected with 30 mg/kg of Evans Blue (EB), ten minutes after the administration colon was washed with 1 ml physiological saline and EB presence was analyzed spectrophotometrically. Animals were euthanized with an over dose of Nembutal® and colon tissue samples were surgically removed and were preserved in formalin for further histopathological analysis.

Spectrophotometrical analysis of the saline fluid collected from the colon showed no traces of EB, indicating that plasma did not affect the tissue integrity. Histology analysis also showed that no macroscopic damage was induced to the colon tissues by plasma treatment or probe manipulation (Figure 7).



FIGURE 6 Photograph of cold spark plasma inside of the mouse colon: the colon here is not punctured and the light generated by plasma is seen through the thin tissue.

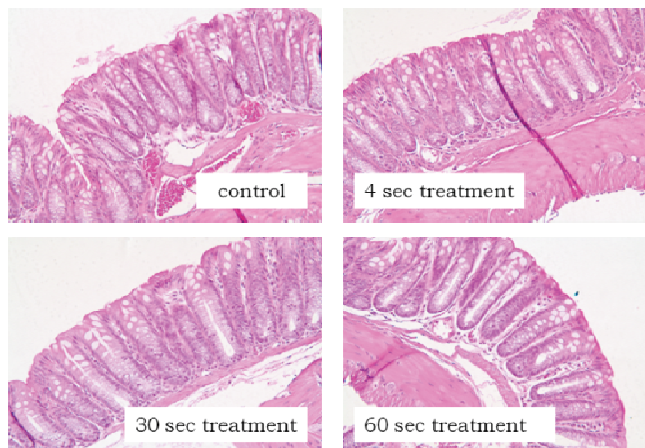


FIGURE 7 Colon tissue histology shows no detectable damage

B. DSS model Disease Progression After Plasma Treatment

The next step was to check the response of the disease progression to plasma treatment; hence 24 animals were divided into 4 groups each receiving 0, 4, 30 or 60 seconds of plasma treatment every alternate day for 7 days. All animals were fed 2.5% DSS for 7 days in parallel with plasma treatment. DAI was scored everyday to see which dosage of plasma was most effective in controlling the progression of the disease. For the plasma probe to be inserted and to go through the colon, the colon has to be cleansed of stool specks. To do so, the animals were fed a polyethylene glycol based laxative along with DSS one day before plasma treatment. However, the stool consistency on the next day was compromised as the laxative made the stool consistently loose. Hence, data extracted from the study were bifurcated and were analyzed using a three pronged approach considering:

- a. Weight, stool consistency, and presence of blood in the stool.
- b. Weight, and presence of blood in the stool (without stool data)
- c. Mean hemocult (visible blood in the stool)

Results of the experiment (Figure 8) show that, in the control group the DAI scored using weight, stool consistency, and blood visibility in the stool reaches a value of 2.3 by the end of 7 days. The group which received 60 seconds of plasma treatment reached a DAI of 1.6 on day 7. Hence, the disease is not controlled very well in the 60 second plasma group; on days 3, 4 and 5 one minute of plasma treatment stabilized the disease and prevented it from progression. However, as the severity of the disease increased the plasma treatment effectiveness ceased. 4 seconds of treatment resulted in disease reduction during the end stage of the study and it is comparatively better than the 60 second

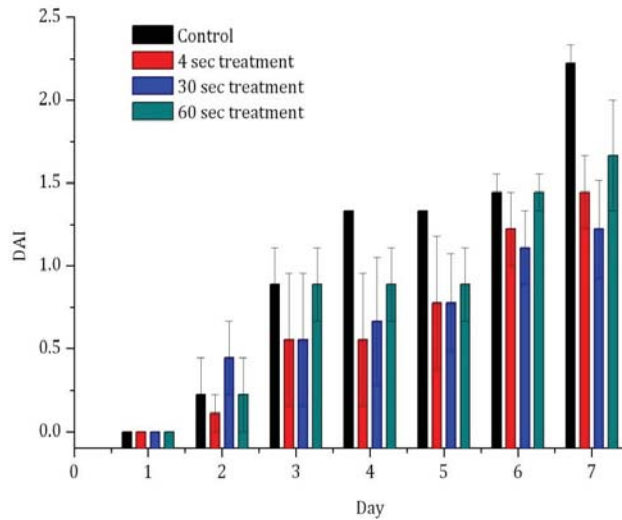


FIGURE 8 Progress of colitis, mean DAI: results of the second stage of the study (considering weight, stool consistency and hemmocult)

treatment. Here the disease was well controlled through the course of the study. The most efficient dosage of plasma as shown by the study is 30 seconds exposure. This group showed high resilience against the progression of the disease, and as compared to the control, showed great ability in stopping the progression of the disease even when DSS is being fed in parallel. The maximum DAI in this group was 1.2 as observed on day 7, the day of maximum disease prevalence.

The laxative that was administered to the animals on days prior to plasma treatment might have compromised the score based on stool consistency as this makes the stool comparatively loose, and therefore on Figure 9 only hemocult data have been considered as one of expected effects of plasma treatment is blood coagulation inside the inflamed colon. As the prime facet of tissue degradation of colitis is through bleeding out of the tissue which can be observed on the stool study. Hence this data is critical to check if plasma can actually clot and coagulate the blood inside the colon. Data shows that a statistically significant difference was obtained between untreated animals in control group and animals treated with plasma for 30 seconds. On the final days of study hemocult reached DAI of 4 in the control group while the “30 seconds” group had a DAI of just over 0.5. It can be inferred from the data that the “30 seconds” and “4 seconds” groups are showing blood coagulation inside the colon.

C. Comparison of Effectiveness of Plasma Treatment With Conservative Therapy of Ulcerative Colitis

The goal of the last stage of the study was to investigate the effectiveness of plasma treatment as an adjuvant to conventional antioxidant drug (5-amino salicylic acid (5-

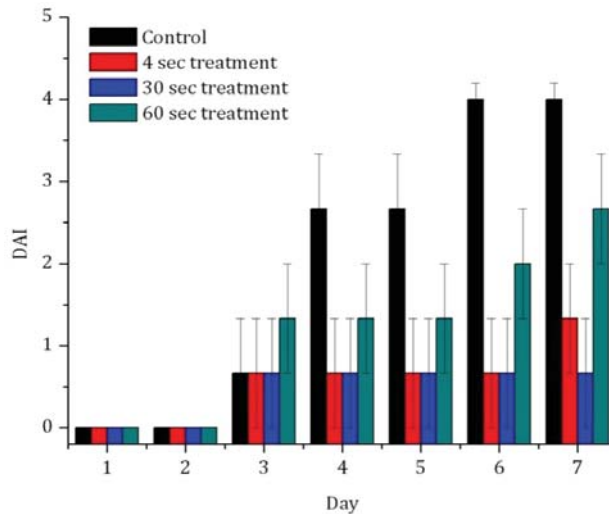


FIGURE 9 Progress of colitis, mean DAI: results of the second stage of the study (considering hemocult only)

ASA)) treatment. Based on previous results, where it was shown that 30 seconds of plasma treatment gives the best results in controlling and reducing the disease progression compared to all other groups, this treatment dose was selected for the next step. In this set of experiments 24 animals were divided into 4 groups: control group 1, where no plasma or drug treatment was performed, and groups where mice were treated either with plasma alone (group 2), 5-ASA alone (group 3), or drug and plasma together (group 4). All four groups of animals were fed with DSS for six days. On days 2, 4, and 6 they received 2.5 % DSS dissolved in water and for days 1, 3, and 5 (one day before plasma treatment) they received 2.5% DSS dissolved in 15% poly ethylene glycol based laxative to clean the colon. Plasma probe was introduced into the colon 4 cm from anal verge in groups 2 and 4, where group 2 received 30 second dose of plasma treatment only, while group 4 received same dose of plasma treatment in together with 0.1 ml of 5-ASA treatment. Animals in groups 3 and 4 were treated with 0.1 ml of 5-ASA. The DAI was scored everyday during the tenure of the study. On the seventh day of DSS treatment and final plasma treatment DAI was measured and animals were euthanized with an over dose of Nembutal®.

The disease progression for all four experimental groups is shown on Figure 10. The control group showed a steady increase through the course of seven days with the DAI reaching 2.7 on day 7. The plasma treatment group was administered 30-second plasma treatment and obtained data are in direct correlation with the data acquired in the previous study and also show a constant increase of disease progression. However, the disease curtailed to near 1.8 on the DAI scale. The group 4 showed very positive results as both in combination proved to be effective in controlling the disease and keeping the DAI on a level of 1.2 on the DAI scale.

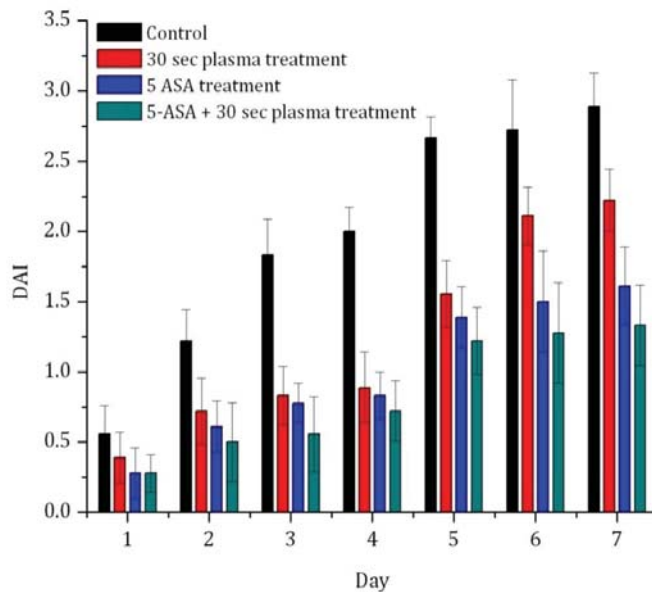


FIGURE 10 Progress of colitis, mean DAI: results of the third stage of the study (considering weight, stool consistency and hemmocult)

Figure 11 is designed to express the mean hemoccult charted on a scale of 0 to 4 with 4 being the advanced stage of the disease. The plasma treatment resulted in a significant drop on the hemoccult scale as compared to the control. This shows that plasma actually was working in clotting the blood that was being pumped out of the inflamed region in the colon. The combination treatment further reduced the hemoccult score as a two pronged approach is taken against the disease.

The most important aspect of the study is to verify the hypothesis that plasma can be used as an add-on to the conventional drug treatment to cure the disease faster and with renewed vigor. Experimental results show that in the group which received combination treatment, disease progression was further decreased as compared to the groups which received the drug or plasma alone (Figure 10). The bars show a statistical significance in the two groups as plasma and 5-ASA reduce the disease as compared to the control group on days 5 and 7.

The results of our first invasive in-vivo experiments of cold plasma treatment of colon tissue in case of ulcerative colitis mouse model show that plasma can significantly reduce severity of the disease progression. In fact, while untreated (control) animals undergo weight loss, loss of stool consistency, and excessive bleeding, showing disease progression up to DAI number 3.1 (when 4 corresponds to lethal stage of the disease), plasma treated ones appeared to have significantly lower disease progression rate with reduced reaction to inflammation.

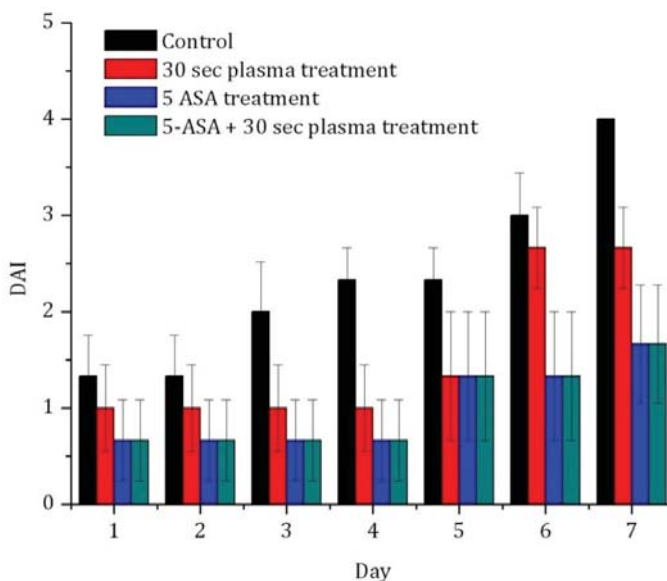


FIGURE 11 Progress of colitis, mean DAI: results of the third stage of the study (considering hemocult only)

Cold plasma treatment apparently has positive effect on inflamed colon tissue when applied for ulcerative colitis treatment. First, analyzing contribution of hemocult level to the DAI, it is shown that plasma may cause effective blood coagulation in colon ulcers. Indeed, Fridman et al. have experimentally shown that treatment of normal whole blood both in in-vitro and in-vivo cases with floating electrode dielectric barrier discharge (FE-DBD) plasma leads to initiation of natural coagulation cascade followed by fast, on the order of seconds, formation of blood clot^{10,28}. However, contribution of various plasma species and detailed mechanism of this effect remain unclear.

Another possible effect of cold spark plasma is related to nitric oxide production. It is well known today, that NO plays an important role in wound healing and tissue regeneration by regulation of blood vessel tone and blood coagulation, immune system and early apoptosis, etc.²⁸ Exogenic delivery of NO-donors (compounds that contain and release or synthesize nitric oxide upon contact with tissue) to the wound promotes and speeds up healing processes²⁸. At the same time, overdosage of NO is known to have cytotoxic effect, however exact concentrations of exogenic NO that provide either healing of “killing” effects are not yet established²⁸⁻³¹, especially, for gaseous NO.

Additionally, the role of NO may be related to “deactivation” of oxidants with formation of acidic environment. One possible explanation of pathogenesis of ulcerative colitis is given by so-called “Radical Induction Theory”, which suggests that the main cause of inflammation is excess un-neutralized H_2O_2 ³⁶. Hydrogen peroxide is produced within colonic epithelial cells, and then easily diffuses through cell membranes, and

through formation of highly reactive hydroxyl radical extensively causes oxidative damage to the cells. In this situation, increased acidity may play an important role in process of inhibition of inflammation: currently used drugs, for example, 5-ASA, which is acid, probably acts as an “antioxidant”. To check this hypothesis, we have treated 100 μl of H_2O_2 water solution with initial concentration of about 30 mg/l with spark discharge plasma (Figure 12). In order to simulate closed environment of the treated colon, discharge cell and solution holder were incased using tygon tube.

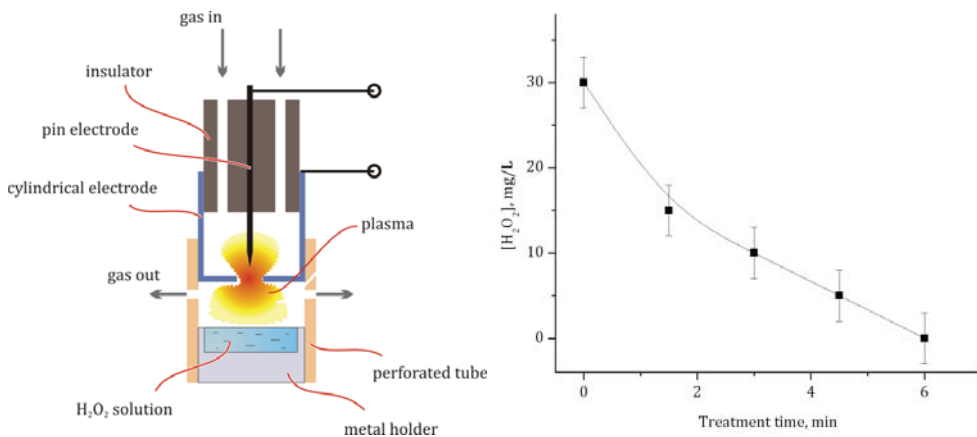
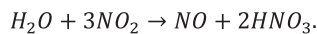
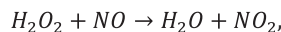


FIGURE 12 Scheme of the experimental setup and the results of hydrogen peroxide PHD plasma treatment

The result of the experiment shows that hydrogen peroxide was decomposed completely within 6 minutes of treatment. Similar effect may appear significantly faster in the colon tissues, since amount of treated liquid in this case is much lower. Possible mechanism of the destruction may be related to chemical reaction of H_2O_2 with NO with formation of nitric acid:



IV. CONCLUSIONS

Experimental study presented in this paper points to a possibility of internal cold plasma treatment of inflammatory disease model — a DSS mouse model of ulcerative colitis. Major results presented may be summarized as follows:

1. In the first stage of the plasma study, the primary goal was to understand the interaction of cold plasma with a living animal. The study showed that the cold plasma alone in graded doses did not cause any damage to

the colonic tissue or the animal based on Evan's Blue extravasation and histological analysis.

2. The second stage of the plasma study posed the challenge of ascertaining the graded dose at which the progress of the disease (ulcerative colitis) can be controlled. It is concluded that plasma did not adversely affect the animals and did not increase the progress of the disease. Surprisingly, it reduced the disease from progressing rapidly as compared to the control. The exact mechanism by which cold plasma induced its beneficial effect remains unknown.
3. The final stage of the plasma study was designed to evaluate and check the activity of plasma as an adjuvant to the industry standard drug 5-ASA in controlling the disease. Combination therapy of 5-ASA and cold plasma showed that there is a significant therapeutic relevance when it comes to adding plasma to the drug in controlling colitis in the DSS model during the induction phase.
4. Possible explanations of observed positive effects of plasma treatment were proposed. First, it was noticed, that plasma treatment caused significant decrease of blood oozing from ulcerated colon tissues. Also, reduced progression rate of the disease may be related to "antioxidant" and "healing" effects of plasma produced nitric oxide.

Although presented results are very promising, many questions remain unanswered and further development of the model and accurate extensive study are both needed. First, possible adverse long-term effects of plasma treatment should be carefully investigated. Another set of questions is related to mechanisms of plasma interaction with tissues, including effects of NO, UV, temperature, acidity, other active neutral and charged plasma components. However, this study clearly demonstrates feasibility that the cold plasma treatment may be safely applied not only for sterilization of living tissues, but also to achieve a medically relevant therapeutic effect(s), which opens new possibilities of cold plasma applications in the field of medicine.

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