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Effects of Nonthermal Atmospheric-Pressure Plasma on *Drosophila* Development

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ABSTRACT: Nonthermal atmospheric-pressure plasma (NTAPP) is known to induce a wide range of responses at the cellular level. This study is concerned with the effects of NTAPP on a eukaryotic organism as a whole: *Drosophila melanogaster*. Exposure influenced the larval viability and caused an array of traits that can be classified into three major groups: (1) phenotypic anomalies in larvae (such as melanotic masses, melanized and broken trachea, incomplete shedding of the old cuticle during molting), morphological anomalies of pupae (small size, abnormal form, aberrant development, cryptocephalic forms), and developmental anomalies in adults (abnormal formation of wing, legs, and thorax); (2) larval behavior alteration (nonfeeding of first and second instar larvae, premature wandering, running away from food, immature pupae formation); and (3) excessive fat accumulation and lipid oxidation. The majority of the observed traits can be linked to molting and metamorphosis controlled by the endocrine system, in particular with the steroid hormone ecdysone. Results support the hypothesis that the interaction of NTAPP with the membranes of various organs can have a major role in the interruption of normal ecdysogenesis.

KEY WORDS: biomedical applications of plasmas, mortality, larval development, melanism/melanotic masses, endocrine system, immune system

INTRODUCTION

Exposure to nonthermal atmospheric pressure plasma (NTAPP) has been shown to induce a broad array of biological responses. At the cellular level, there is evidence of that NTAPP can induce apoptosis,[1] membrane poration,[2] integrin activation,[3] DNA damage,[4] platelet activation,[5] fibroblast activation,[6] increased cell proliferation,[6] and cell cycle–dependent cell death.[7] At the tissue level, NTAPP has been associated with tissue regeneration,[8] angiogenesis,[9] and selective, dose-dependent reduction in tumor size.[7,10,15]

NTAPP generates reactive oxygen species (ROS), reactive nitrogen species (RNS), and ultraviolet (UV) radiation. ROS are thought to be the main contributors to the previously described results.[11] The exact mechanism through which these radicals alter the cell is still under intense debate. Several studies have reported membrane modifications such as lipid peroxidation[26] and transmembrane protein conformational changes.[3,27] These suggest that the cell membrane is key in the NTAPP-induced activation of intracellular signaling pathways[12] culminating in the initiation of DNA damage.

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Several NTAPP generators are already in clinical use, in particular, for the treatment of chronic diabetes wounds[13] and tumor bed exposure following surgical glial tumor removal.[14] Yet, the overwhelming complexity of biological systems is such that it is imperative to further understand the mechanisms of interaction of nonequilibrium gas discharges with living organisms, tissues, and cells.

The biological similarities between the fruit fly and more complex species such as humans have turned *Drosophila* into an appropriate model to better understand the basis of many human diseases and disorders, such as obesity, hormone regulation, and autoimmune disorders.[16,17] This study reports the first results on the effects of NTAPP on the development of *Drosophila* larvae. We show that NTAPPs induce an array of outcomes (most commonly cell death, ecdysogenesis, and melanotic tumors) and discuss their possible causes.

II. MATERIAL AND METHODS

A. Experimental Apparatus

The NTAPP was generated by means of a dielectric barrier discharge (DBD). The electrodes were 1.5 mm thick copper slabs, the dielectrics were 0.5 mm thick mica slabs, a 2.5 mm acrylic spacer with a 30 × 30 mm square aperture set a discharge gap volume of approximately 2 cm^3. The discharge was powered by a pulse high voltage source that is similar to the one described by Fridman et al.[5] and shown in Figure 1. Voltage pulses were shaped as damped sinusoidal oscillations with a maximum amplitude of 1.1 kV (peak to peak), period of 17 μsec, decay time of 23 μsec, and repetition time of 1 msec. Typical oscillograms of voltage and current are shown in Figure 2. The average deposited power was estimated to be around 3.3 W; hence, a 10 sec exposure produced a total electrical dose of 3.7 J/cm^2. The discharge operated in air at room temperature. The UV-NIR emission spectrum (measured with an Ocean Optics HR4000 spectrometer [Winter Park, Florida, USA]) showed a significant presence of RNS (see Fig. 3) but no measurable ROS.

Two different delivery setups were devised in order to determine the relative importance of UV radiation compared to that of plasma reactive species. In one setup, the subjects were directly exposed to the NTAPP inside the gap volume. In another, air

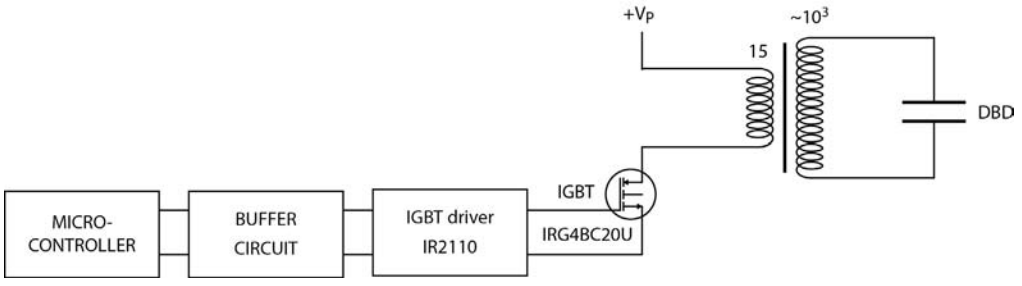

FIG. 1: Discharge circuit schematic.

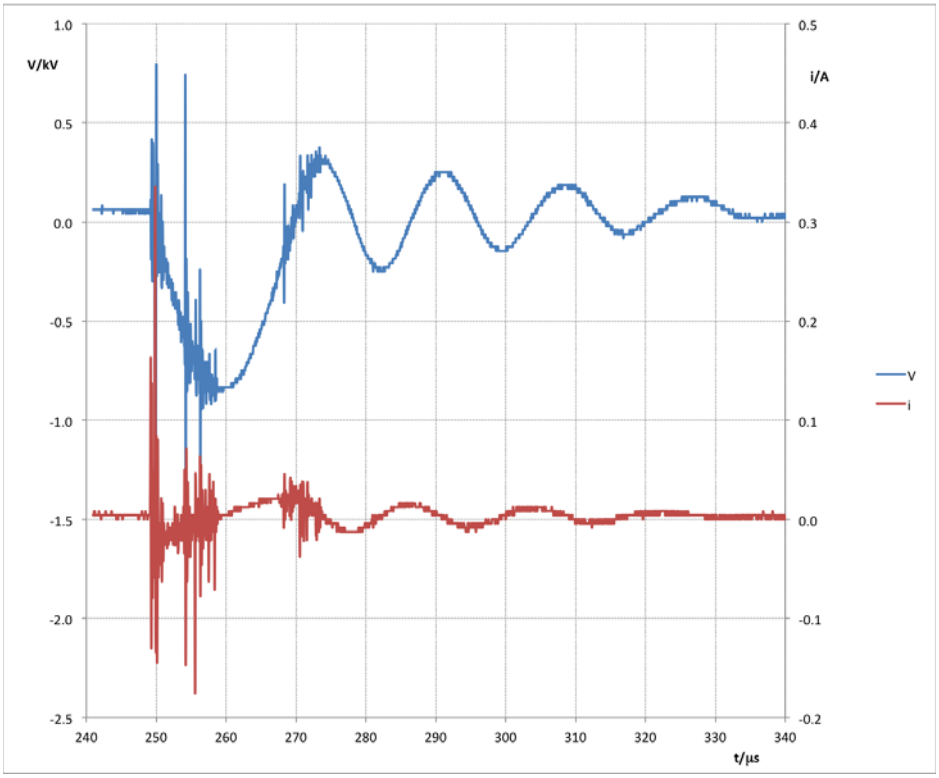

FIG. 2: Discharge voltage and current oscillograms.

was pumped through the discharge gap at a flow rate of 25 L/min, and the larvae were exposed to the plasma jet at a distance of 15 mm. In the latter case, the larvae were not inside the field of the discharge and therefore were not directly exposed to UV radiation.

B. Fly Strains

Two strains were used: (1) standard laboratory strain from "Laboratori Fabra," courtesy Department of Genetics, University of Barcelona; and (2) wild-type strain that was obtained after collecting female flies on the university campus, establishing isofemale lines, and performing sib-mating for six successive generations before exposing one of the established lines. Flies were raised at 24°C on standard cornmeal and sugar media.

C. Exposure

It was found that the minimum exposure time for the observation of a sizeable biological effect was 60 sec in a direct exposure and 120 sec in an indirect exposure (jet). These exposure times were used for all experiments reported here.

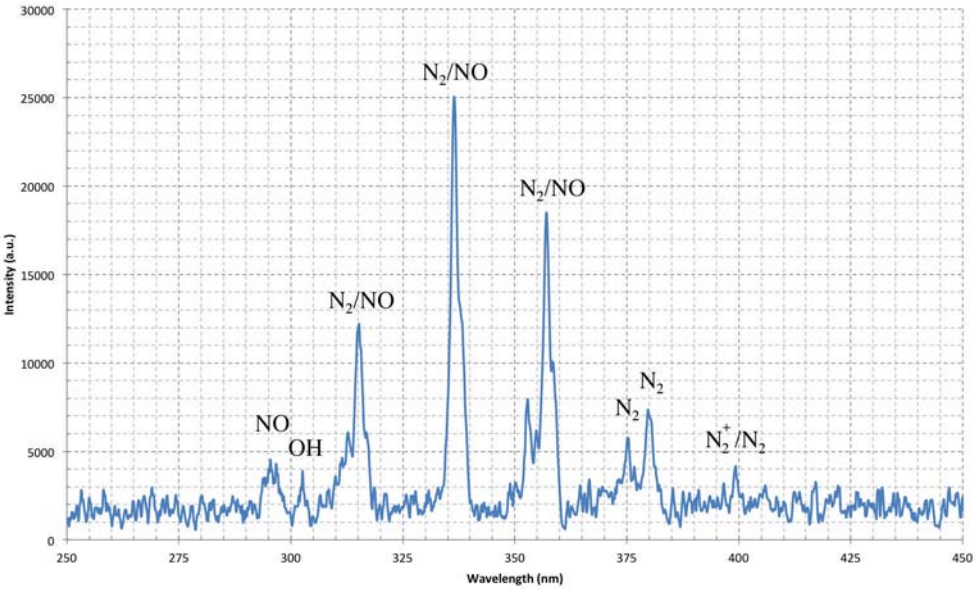

FIG. 3: Discharge UV-NIR emission spectrum.

Larval staging was performed in accordance with standard criteria.[28]

The larvae of the same stage were kept at 4°C for 15 min in order to render them less active. Subsequently they were exposed and then kept in groups of 10 in a Petri dish containing standard media. They were observed under stereoscope within 1 and 4 hours after exposure. Then the larvae were observed and transferred to a new dish every 24 hours to ensure absence of fungi. The dead larvae were mounted and observed under microscope. Those with anomalies (morphological or behavioral) were transferred to a separate Petri dish. The larvae were observed until they either died or reached adulthood. The dead pupae were dissected and mounted on slide preparations. The emerged adults were observed morphologically and kept in new vials. Since no differences were detected between the two lines employed, they are both referred to as *Drosophila melanogaster* throughout the text.

Statistical tests were performed by chi-square analysis, and the level of significance was determined after Bonferroni correction.

III. RESULTS

A. Mortality and Melanism

After exposure, 55.5% (95 out of 171) of the first instar larvae, 27.6% (45 out of 163) of the second instar larvae, and 26.9% (28 out of 104) of the third instar larvae died within 24 hours. The mortality rate of the first stage larvae was not significantly different (χ^2 = 1.110, 1 *df* = 1; *P* = 0.292) from the control; therefore, other factor(s) such as mechani-

cal manipulation might have caused the mortality because the larvae are very fragile at this stage. On the other hand, the exposure had a significant effect on the mortality of the second and especially third stage larvae ($\chi^2 = 6.595$, $df = 1$; $P = 0.0102$; and $\chi^2 = 30.907$, $df = 1$; $P = <5 \times 10^{-5}$, respectively) compared with the control. The most common phenotypic trait in the larvae after exposure was the appearance of melanism/melanotic masses (MMs). This trait could appear rapidly, as soon as 30 min after exposure, or later throughout the developmental stages (Fig. 4b, c). The mortality rate was significantly higher in larvae, regardless of the stage, that had developed MMs compared to others (supplementary data). Many of the dead larvae showed a disintegration of the fat body, and their body cavity was filled with free lipid droplets of different shades that could be related to different stages of lipid oxidation (Fig. 4d).

MMs occurred either in a restrained manner (constant size and number) or they became uncontrolled, increasing in size and number. Evolution of MMs over time was recorded in photographs. Overall the MMs in the head and the thoracic segments caused larvae death. However, when they occurred in other segments, the larval viability and mortality depended on the size and number of the MMs. Some larvae had melanized anterior or posterior spiracles. Those with anterior melanized spiracles died, but the ones with posterior melanized spiracles normally survived, because it mainly occurred in the cuticules that were later detached during ecdysis. Four of the 279 control larvae also developed small and localized MMs in the hemolymph without alteration in their survival.

This anomaly was also observed in pupal stages (Figs. 4 and 5), and (as in larvae) widespread melanism was associated with high mortality of the pupae. The localized MMs did not show any effect on pupal viability. The adults formed by these latter pupae could either carry free small MMs in their abdomen or had melanism in body parts (legs, internal organs).

B. Behavior and Ecdysis

After exposure, regardless of the stage, the abnormal larvae lacked normal feeding behavior and either ran away or stayed on the food surface, slowly rotating their head. Some larvae presented broken lateral or main tracheae and inevitably died after 2 or 3 days. Others continued with the abnormal behavior up to 30 days without ever reaching the normal larvae size of the prepupal stage. Their body remained small and compact with curved trachea. These larvae either finally died, presenting signs of ecdysis failure (such as simultaneous presence of the third and second instar mouthparts, anterior spiracles, and cuticles in the same animal), or they managed to form premature pupae with larval shape and a soft puparium without cuticle darkening and arrest in development (Fig. 4).

The larvae that formed normal pupae could present defects later in pupal ecdysis, head eversion leading to a cryptocephalic dead pharate adult. Some flies were able to eclose, but those with failure in expansions of the thorax or thoracic appendages (legs and wings) only survived for a few days (Fig. 5c, g).

FIG. 4: Anomalies detected in larvae and pupae after exposure to NTAPP. (a) Control larvae; (b) larvae with several internal melanotic masses scattered throughout the body; (c) compressed larva, with twisted trachea and internal and subcuticular melanotic masses; (d) larva with molting defects; presents a second and third stage spiracle (1), broken trachea (2) and disintegration of fat body (3); (e) X 20 magnified image of the top of the head of the larva in (d). Displays retaining of the old cuticle (1), the characteristic second and third stage spiracle (2 and 3), and duplication of mouth hooks (4, 5); (f, g) control pupae; (h, i) early pupae failed in eversion of the anterior spiracle, retention of larval form and internal and subcuticular melanotic masses; (j) pupae presenting internal melanotic masses.

FIG. 5: Anomalies observed in pupae and adults resulting from larvae exposed to NTAPP (a, b) dissected control pupae; (c) dissected cryptocephalic pupae with two pigmented eyes in the abdominal cavity, indicated by black arrow; (d) cryptocephalic pupae where it is observed one eye developed in the abdomen (should be in 1 and is in 2); (e) control adult; (f) adult with the formation and segmentation of three pairs of legs, completely changed; (g) adult with abnormalities in the formation of the thorax, indicated by the black arrow.

IV. DISCUSSION

Intrinsically ROS are produced by some cell organelles as part of normal metabolism and are also known to play important role in cell and tissue signaling. However, excessive ROS production targets essential macromolecules and organelles, damaging the cells irreversibly. In the present study, larval death was the first outcome of NTAPP exposure and occurred within a few hours to a few days or even weeks after treatments. ROS generated by NTAPP could have caused severe oxidative stress resulting in larvae death.

Other anomalies can be mainly associated with the two major and interrelated players during *Drosophila* development: immune system function and ecdysogenesis.

A. Immune System

Different forms of melanism (floating melanotic masses in the hemolymph; melanisation of cuticle and internal organs) observed in the present work could be related to the activation of the immune system. The melanization reaction is a major immune response in *Drosophila* to contain a microbial infection or/and facilitate wound healing by producing melanin at the proper site.[18–20] This process involves rapid synthesis of melanin by phenol oxidase conversion. Another type of defense leading to melanism is humoral reactions that activate proteolytic cascades and produce antimicrobial peptides (AMP), mainly by a major immune-responsive tissue, the fat body. The defects in the immune system (cellular or humoral) can lead to the formation of melanotic tumors.[21] The basement membrane (BM) has an essential role in preventing autoimmune activation in *Drosophila*.[19] In a mutant strain with melanotic tumors, the disintegration of the BM of the caudal fat body prior to encapsulation and melanization has been reported.[20] We suggest that the floating melanotic masses observed in this work could be related to the disintegration of fat body cells, their encapsulation, and melanization. Also, the NTAPP exposure could have physically damaged the cuticle and the epidermis, releasing damage signals that attract hemocytes to the damaged area. The subsequent clotting and melanization of the area resulted in the appearance the melanized cuticle and epidermis. The melanotic masses have also been detected in *Drosophila* larvae infected with parasites and bacteria.[18,21] None of these agents could be involved in the formation of this trait in the present study because a cocktail of antibiotics was used in rearing the *Drosophila* for six generations. This eliminated any possible bacterial infection. Also, under controlled laboratory conditions the larvae were not exposed to any parasitoid wasps.

The melanization of some organs such as tracheae, Malpighian tubules, and the digestive system, could be associated with activation of humoral or epithelial immunity, after exposure that normally occurs upon microbial infection. However, a significant AMP gene expression level has been reported[22] in the fat body in the absence of infection and in several surface areas in contact with the external environment (such as epidermis of digestive and respiratory tracts). Further research is needed to determine if NTAPP can induce the proteolytic cascade by mimicking microbial infection or other similar

mechanisms. Overactivation of the immune system could have caused the formation of multiple melanotic tumors in the larvae, frequently leading to their death.

B. Ecdysogenesis

The abnormal phenotypes and behavior observed after exposure can be related to anomalies in the ecdysogenesis. Pulses of the steroid hormone ecdysterone (20-hydroxyecdysone [20E]) regulate each of the major developmental transitions in *Drosophila*. The pulse during the first and second larval instars triggers molting of the cuticle that is mainly restricted to the epidermis.[23] At the end of the third instar, a higher titer of the hormone prompts widespread signals that induce prepupal changes, including body shortening, cuticle hardening, leg and wing imaginal discs eversion and elongation. The final ecdysone pulse triggers prepupal to pupal transition, during which the head is everted and final elongation of the legs and wings is achieved. Therefore, there is a striking similarity between the deregulation of ecdysone and observed phenotypic anomalies such as: inability to shed the cuticle, resulting in a third instar larva with second instar cuticle attached in the mouth hook; failure to evert the anterior spiracles; a pupa with an elongated shape (failure to shorten); failure in hardening of the puparium; failure in disc eversions, resulting in cryptocephalic pharate adult with short and distorted legs; and failure in full eversion leading to an adult with internalized wings and half a thorax.

C. NTAAP Possible Interferences with Ecdysogenesis

Exposure could have influenced the normal feeding behavior, thus reducing the uptake of sterols (mainly cholesterol), which are the necessary compound in the biosynthesis of 20E. Development of *Drosophila* larvae reared on cholesterol-free food was arrested in the larval stages.[24,25] Defects in sterol transport and trafficking impaired ecdysterol biosynthesis, which leads to developmental arrest phenotypes similar to those reported here, such as problems with molting and death, prolonged larval stages, and failure to transform into a pupa. Improper spiracles and altered cuticle morphology have been reported in mutants.[25] Also NTAPP could have disrupted the PTTH (prothoracicotropic hormone) signal transduction cascade in the prothoracic gland by interacting with membrane or/and cytosolic lipids. The same phenomenon has been reported in *Drosophila* mutants.[25] Future work at the molecular level will shed light on the nature of the interaction between NTAPP exposure and ecdysogenesis.

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