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FULL PAPER

PLASMA PROCESSES AND POLYMERS

Zebrafish larvae as a toxicity model in plasma medicine

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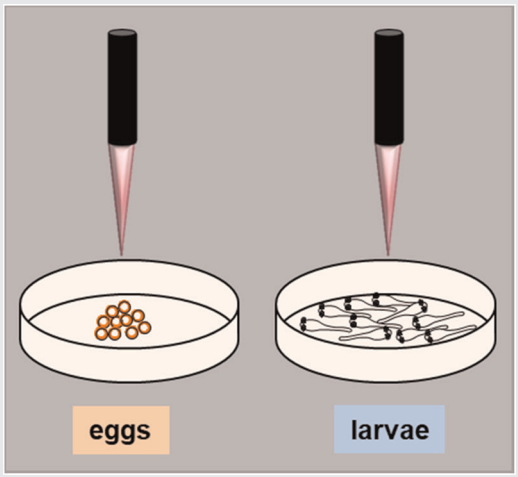
Abstract

Plasma technology has emerged as a promising tool in medicine that, however, requires not only efficacy but also toxicological assessments. Traditional cell culture systems are fast and economical, but they lack in vivo relevance; however, rodent models are highly complex and necessitate extended facilities. Zebrafish larvae bridge this gap, and many larvae can be analyzed in well plates in a single run, giving results in 1–2 days. Using the kINPen, we found plasma exposure to reduce hedging rates and viability in a dose-dependent manner, accompanied with an increase in reactive oxygen species and a decrease of glutathione in plasma-treated fish. Modest growth alterations were also observed. Altogether, zebrafish larvae constitute a fast, reliable, and relevant model for testing the toxicity of plasma sources.



KEYWORDS
glutathione, kINPen, reactive oxygen species, ROS

1 | INTRODUCTION

Eukaryotes have several systems to maintain the cellular redox state at physiological conditions.[1] During pathological conditions such as acute inflammation, the antioxidant mechanisms are overwhelmed, leading to oxidative stress-induced cell death.[2] However, chronic oxidative stress also leads to the progression of several pathological conditions like diabetes,[3] cancer,[4] and neurodegeneration.[5] This duality of reactive oxygen species (ROS) can be harnessed for a favorable outcome specific to disease models. For instance, effective management of inflammatory disorders[6] and hypertension[7] was accomplished by antioxidant therapies. Conversely, pro-oxidant therapies have been employed against macular degeneration[8] and cancer.[9]

Cold physical plasma is a pro-oxidant therapy routinely employed in biomedical applications to promote

wound healing in patients,[10] and there is a tremendous surge to improve its feasibility in antitumor application.[11] Physical plasma is a partially ionized gas that generates a combination of reactive oxygen species (ROS) known to be involved in the oxidation of biomolecules, redox signaling, and regulated cell death.[12] This induces redox imbalance, oxidative stress, and subsequent cytotoxicity in tumor cells.[13,14] Several cellular mechanisms induced by cold plasma have been elucidated in two- and three-dimensional cell culture systems.[15–17] However, these model systems reflect the capacity of living organisms to deal with oxidative stress only to a limited extent. Preclinical rodent models, in turn, replicate the disease and toxic events to a greater extent, but their numbersome use in the screening of large number of conditions or for toxicology studies is discouraged by ethical constraints. Preclinical research, hence, is in need of models that bridge the gap between cell culture and rodent models, such as zebrafish larvae.[18,19]

Zebrafish larvae represent a powerful model in biomedical research due to their clinically relevant disease models, optical properties, *ex-utero* development, and cost-efficient husbandry.[20] Orthotropic tumor xenografts have been reported that have pushed this model for clinical relevance.[21] Zebrafish larvae are also suitable for toxicology studies.[22] They have been previously used for research on ROS as well as oxidative stress,[23] and antioxidant pathways relevant in plasma medicine such as Nrf2 (nuclear factor erythroid 2-related factor 2)[24,25] have also been described in this model.[26] To this end, it seemed natural to investigate the suitability of zebrafish larvae serving as a toxicological model for the effects of plasma treatment. Using the well-characterized plasma source kINPen,[27] in our proof-of-concept study, we found dose-dependent toxicity of plasma treatment concomitant with the ROS increase and decrease of antioxidants. Interestingly, we found the treatment of both the zebrafish larvae and their eggs to respond to plasma treatment, suggesting this model to be not only suitable for toxicity but also for developmental plasma medical research.

2 | MATERIALS AND METHODS

2.1 | Animal care

All experiments were performed according to German animal protection law overseen by the "Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Rostock" of the federal state Mecklenburg, Western Pomerania (Germany). Fresh zebrafish eggs were obtained from wild-type zebrafish bred under standard conditions, as previously described.[28] Eggs were incubated in E3 medium at 26 ± 0.5°C until hatching. At 3 days postfertilization (dpf), embryos were transferred to 96- or 6-well plates for experimentation. All experiments were completed on 4 dpf. For experiments with zebrafish larvae, ethical approval is only required after 5 dpf.[29]

2.2 | Plasma source and ROS detection

In this study, the atmospheric-pressure argon plasma jet kINPen was utilized and operated at two standard liters per minute of argon (99.9999% purity) gas (AirLiquide). This plasma source is well characterized from the physics[27] and the biomedical[30] points of view. The ROS production was assessed by measuring the stable reaction product of short-lived ROS, hydrogen peroxide (H_2O_2). This was done by plasma-treating 1 ml of E3 medium in 24-well plates and quantifying H_2O_2 using the Amplex Ultra Red assay kit (ThermoFisher) against a standard curve, as described before.[31] To assess the antioxidative potential of the E3 medium, the H_2O_2 production of the plasma jet was compared with that generated in phosphate-buffered saline (PBS) alone. The final analysis of H_2O_2 in E3 was performed by dilution in PBS before addition of the detection reagent. The fluorescence was read in a multimode plate reader (F200; Tecan) at λ_{ex} 535 nm and λ_{em} 590 nm.

2.3 | Plasma treatment of zebrafish larvae and eggs

Plasma treatment was done in two different modes. For the treatment mode of fish being inside the medium during the treatment, zebrafish larvae at 3 dpf, with one larva per well, were transferred to a 96-well plate (Eppendorf) in E3 medium (100 µl). The wells were then exposed to different plasma treatment times or the vehicle argon gas alone. This treatment mode was called "medium-covered." For the treatment of eggs, freshly isolated fertilized zebrafish eggs (10 embryos/well) were transferred to a six-well plate in E3 medium. The eggs were evenly distributed with a single layer of eggs. The wells were then exposed to different plasma treatment times or the vehicle argon gas alone.

The second plasma treatment mode was the direct treatment of fish or eggs with the effluent of the kINPen. For the treatment of larvae, the zebrafish

(3-dpf; 40 larvae/condition) were isolated in a cell strainer (40 μm; BD Biosciences). These larvae were then exposed directly to the plasma jet for different durations and immediately after transferred to the E3 medium. The plasma jet treatment was done manually in a meandering fashion, as the treatment of individual fish for several seconds resulted in the drying of the fish and immediate death. This was not only observed for plasma treatment but also with the argon gas treatment alone, pointing to a contribution of the drying due to the gas flux. Hence, manual plasma treatment was performed at a velocity of about 8 mm/s, while frequently dipping the fish or eggs located in the cell strainer into the E3 medium to prevent them from drying during longer exposure times.

The larvae were incubated for 24 h and the percentage of viable larvae was determined visually using the assessment of heartbeat. The eggs were further incubated and the hatching rate and viability were determined microscopically.

2.4 | Live-fish fluorescence imaging

Plasma-treated 4-dpf larvae were incubated with sytox green (2 μM; ThermoFisher), glutathione (GSH)-tracer (2 μM; Tocris), or the ROS indicator CellRox green (2 μM; ThermoFisher) for 30 min. Subsequently, the zebrafish larvae were washed once in E3 medium and incubated with benzocaine (35 mg/L; Sigma Aldrich) for 15 min for sedation. This step was necessary to prevent the motoric activity of the zebrafish larvae in the course of live-organism microscopy. For imaging, a live-cell high content imaging system (Operetta CLS; PerkinElmer) using appropriate excitation/emission wavelengths and a 1.5× (NA 0.03) air objective (Zeiss) was employed. For quantitative image analysis, the zebrafish larvae were segmented and the mean fluorescence intensity was calculated within this region of interest (ROI). The entire image analysis workflow was semiautomatic with algorithm-driven segmentation without any manual user intervention regarding the size or location of the ROI. As software, Harmony 4.9 (PerkinElmer) was used. The determination of the length and width of the embryos was done manually in the software.

2.5 | Single-cell isolation

Zebrafish larvae (40 larvae/condition) were washed twice in Hank's solution (ThermoFisher). Embryos were minced in a Petri dish with a scalpel. The minced tissue was then transferred to a 1.5-ml tube containing trypsin/dispase (0.2%/0.1%) solution and incubated at room temperature for 60 min with constant shaking (300 rpm). The digested tissue was then loaded on a strainer (40 μm; BD Biosciences) and rinsed three times with Hank's solution. The single-cell suspension was washed, and the pellet was dissolved in 1 ml of Hank's solution supplemented with

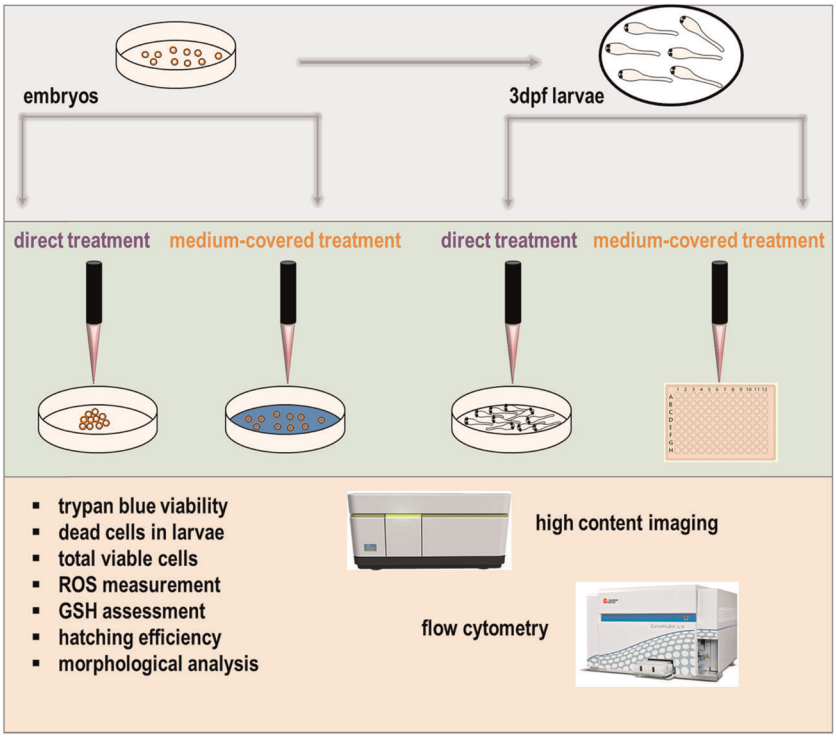


FIGURE 1 Graphical scheme of the study design. Exposure of the zebrafish larvae or zebrafish eggs either directly to cold physical plasma or cold physical plasma in the presence of the culture medium covering the fish or eggs, respectively. GSH, glutathione; ROS, reactive oxygen species

bovine serum albumin (3 mg/ml). The cells were stained with propidium iodide (1 μg/ml) for viability measurements using flow cytometry (Attune Nxt; ThermoFisher). The analysis was done using Kaluza 2.1.1 software (Beckman Coulter). Alternatively, cells from zebrafish larvae previously stained with the ROS tracer CellRox green were added to 96-well plates and subjected to quantitative image analysis to assess the mean fluorescence intensity of individual cells using appropriate software (Harmony 4.9; PerkinElmer).

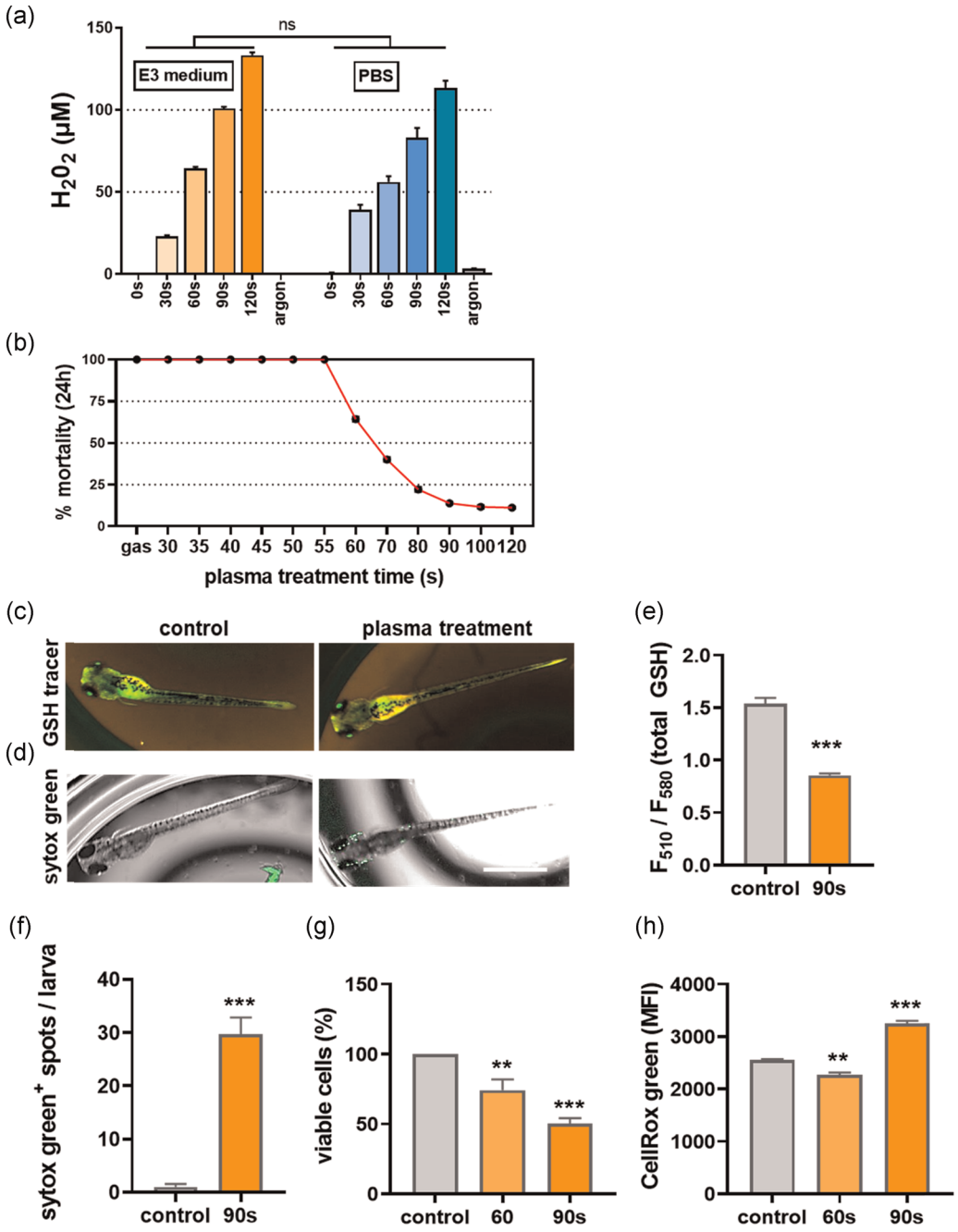


FIGURE 2 The sensitivity of 3-days postfertilization (dpf) zebrafish larvae to medium-covered plasma treatment. (a) Quantification of hydrogen peroxide (H_2O_2) in E3 medium exposed to cold plasma. (b) The mortality rate of 3-dpf zebrafish larvae 24 h after medium-covered plasma treatment. (c–f) Representative overlay (brightfield and fluorescence) images and quantification of 4-dpf zebrafish larvae loaded with ratiometric GSH tracer (c,e) and sytox green (d,f) after exposure to 90 s of medium-covered plasma treatment. (g,h) Viability (g) and the total reactive oxygen species (ROS) levels (h) after enzymatic digestion of zebrafish larvae, as analyzed by flow cytometry and single-cell imaging. Scale bar = 1 mm. Data are mean ± SEM derived from three independent experiments ($n = 30$ larvae per condition). Significance was determined by one-way analysis of variance with Tukey's posttest for multiple comparisons or a two-tailed t test. *$p < .05$, **$p < .01$, and ***$p < .001$

3 | RESULTS

3.1 | Plasma treatment of 3-dpf zebrafish larvae

To investigate the suitability of zebrafish larvae as a model to study the effects of cold physical plasma in living organisms, different treatment approaches, and assay systems were employed (Figure 1). To verify the production of ROS by the plasma jet, the levels of the stable secondary reaction product H_2O_2 were measured in the E3 media after exposure to plasma. Multiple plasma treatment times were investigated, showing a dose-dependent increase in H_2O_2 concentration after plasma exposure (Figure 2a). The plasma-generated H_2O_2 in E3 medium was similar to that generated in PBS alone, suggesting E3 medium to not have a notably high antioxidative capacity. To determine the viability of zebrafish larvae (3-dpf) covered with medium (E3 media) after plasma treatment, 35 larvae per condition were treated for 5–120 s. Twenty-four hours later, the zebrafish larvae were examined microscopically for their viability using the assessment of heartbeat. The results indicated that the larvae showed considerable tolerance to plasma treatment up to 55 s, but almost 50% of the larvae did not survive the 65-s exposure (Figure 2b). For imaging analysis of the anesthetized larvae, the fish were incubated with either a fluorescent GSH tracer (Figure 2c) or sytox green indicating terminally dead cells (Figure 2d) and exposed to either plasma (90 s) or left untreated. The quantitative image analysis of the GSH content in fish after plasma treatment revealed significantly diminished endogenous GSH levels (Figure 2e) and an increased presence of dead cells (Figure 2f) in zebrafish larvae. The zebrafish larvae were then enzymatically digested to generate single-cell suspension, and the viability of individual cells was determined using flow cytometry (Figure 2g). Alternatively, the total intracellular ROS levels of individual cells were quantified using high content imaging microscopy (Figure 2h). A modest increase of terminally dead cells and significantly elevated ROS levels were observed.

Next, the effect of direct plasma treatment on zebrafish larvae (3-dpf) was determined. The difference with the medium-covered plasma treatment was the absence of a bulk liquid on top of the zebrafish larvae during plasma treatment. Instead, the fish were directly treated with the plasma treatment, while maintaining a liquid film to prevent excessive drying. The zebrafish larvae (50 larvae/condition) were plasma-treated on a cell strainer at the indicated intervals (10–90 s). The viability determined after 24 h revealed that the larvae tolerated 30 s of direct plasma exposure to some extent, whereas death was more pronounced with treatment times of 60 and 90 s, respectively (Figure 3a). Similar to the medium-covered plasma condition, zebrafish larvae were incubated with GSH tracer (Figure 3b) or sytox green (Figure 3c) and exposed to plasma or were left untreated. The GSH tracer staining revealed increased oxidative stress (Figure 3d), and the sytox green imaging markedly elevated cytotoxicity (Figure 3e) at 60 s of direct plasma treatment. These larvae were then enzymatically digested to obtain single-cell suspensions. Individual cell viability was determined using flow cytometry (Figure 3f). Alternatively, ROS in individual cells were measured using quantitative microscopy (Figure 3g). A markedly elevated cytotoxicity concomitant with increased ROS production was observed.

3.2 | Plasma treatment of zebrafish embryos

Extended plasma treatment of zebrafish larvae caused an elevation of ROS, a decrease of the antioxidant GSH, and an increased number of dead cells resulting in overall more dead embryos. We sought to assess next whether plasma treatment of the unhatched eggs would impair zebrafish hatching and viability later. This is interesting because the eggshell is reasonably stiff and constitutes an effective shield against environmental insults. Nevertheless, it is known that processes within the eggshell are also subject to redox signaling and increased stiffness.[32] Hence, we explored the number of hatching embryos as well as their morphology (Figure 4a) after medium-covered plasma treatment and direct plasma treatment of the eggs. To our surprise, the former reduced hatching rates already at 30-s plasma treatment (Figure 4b), an exposure time that did not affect the zebrafish larvae's viability. Longer plasma treatment times further decreased hatching rates. However, the morphology of the embryos in terms of length (Figure 4c) and length-to-width-ratio (Figure 4d) was not affected in the plasma conditions and the embryos that did hatch. For the direct plasma treatment, the decline in hatching was overall similar (Figure 4e), albeit the plasma treatment times of the two different modes cannot be directly compared. The length of the embryos that hatched despite plasma treatment was not affected (Figure 4f), whereas the length-to-width-ratio declined modestly but significantly (Figure 4g). Finally, the surviving 4-dpf zebrafish larvae that had hatched from the eggs exposed to plasma were

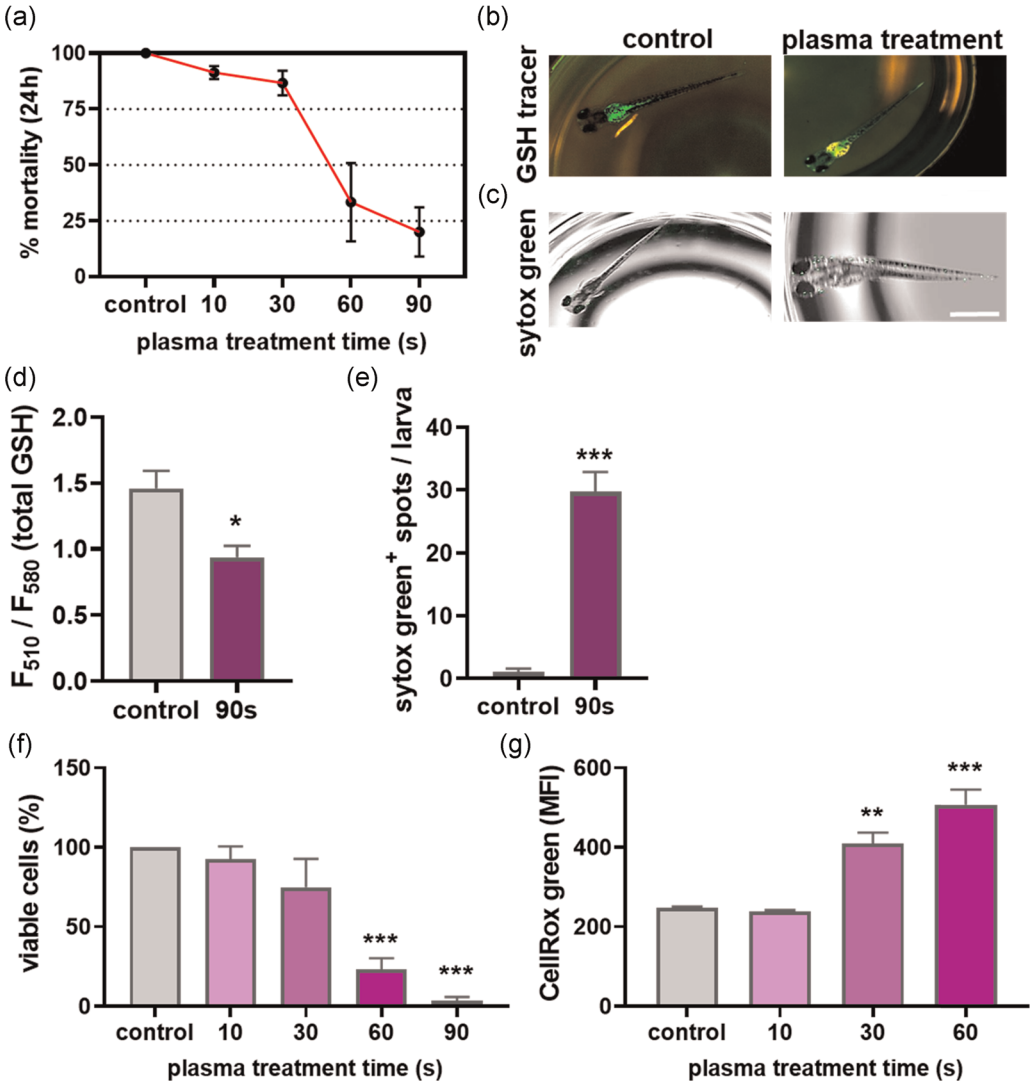


FIGURE 3 The sensitivity of 3-days postfertilization (dpf) zebrafish larvae to direct plasma treatment. (a) The mortality rate of 3-dpf zebrafish larvae 24 h after direct cold plasma treatment. (b–e) Representative overlay (brightfield and fluorescence) images and their quantification of 4-dpf zebrafish larvae loaded either with ratiometric glutathione (GSH) tracer (b,d) or sytox green (c,e) after exposure to 60 s of direct plasma treatment. (f,g) Viability (f) and the total reactive oxygen species (ROS) levels (g) after enzymatic digestion of zebrafish larvae, as analyzed by flow cytometry and single-cell imaging. Scale = 1 mm. Data are mean ± SEM derived from three independent experiments ($n = 30$ larvae per condition). Significance was determined by one-way analysis of variance with Tukey's posttest for multiple comparisons or a two-tailed t test. *$p < .05$, **$p < .01$, and ***$p < .001$

analyzed for any morphological changes (Figure 4h). A small fraction of the larvae had morphological defects such as hypopigmentation at shorter plasma treatment times, and embryonic lethality and spinal defects were observed in a few fish at longer plasma treatment times.

4 | DISCUSSION

Therapy with cold physical plasmas shows beneficial effects in dermatology,[33,34] especially in wound healing.[10,35] With emergence of novel therapeutic applications and plasma source concepts,[36–38] there is an increasing need not only to show treatment efficacy but also to assess toxicological aspects. In this study, we aimed at investigating the suitability of zebrafish larvae as a toxicological model in plasma medicine.

Zebrafish is a frequently used model in toxicity evaluations[39] and human diseases[21] or to understand the function of novel genes.[40] Cold plasma technology produced a multitude of ROS that specifically alter the redox balance[41] and gene expression profiles.[42] In the view of emerging plasma therapies, as demonstrated in clinical case studies in the palliation of head and neck cancer patients in Greifswald,[43,44] a current debate is how to achieve an optimal antitumor ROS cocktail

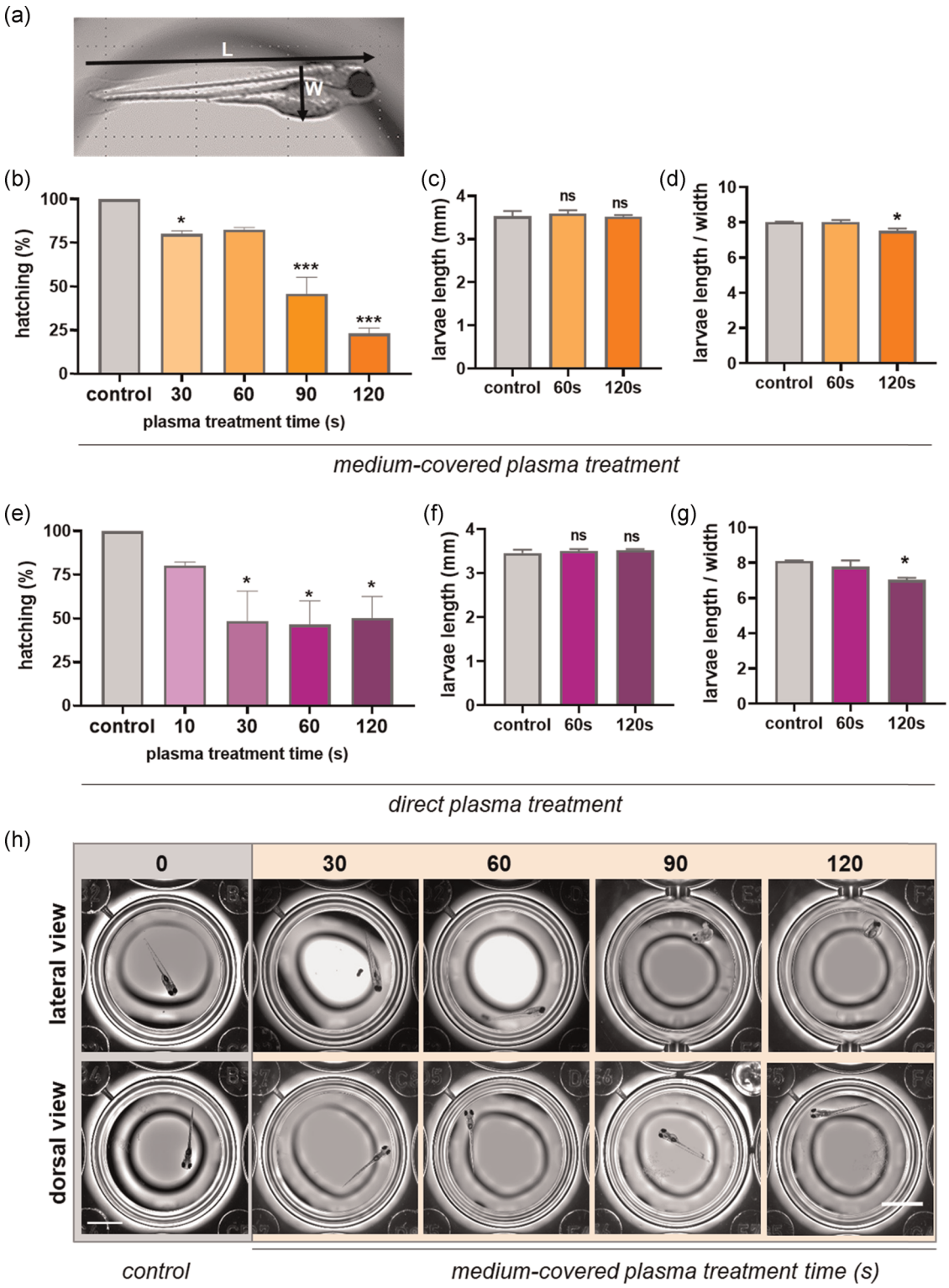


FIGURE 4 The sensitivity of zebrafish eggs and morphological alterations in zebrafish larvae after plasma treatment. (a) A representative image of size determination of zebrafish larvae. (b) The larvae hatching rate of fertilized zebrafish eggs 48 h after exposure to medium-covered plasma treatment. (c,d) Length (c) and length-to-width-ratio (d) of 4-days postfertilization (dpf) larvae after medium-covered plasma treatment. (e) The hatching rate of fertilized zebrafish eggs 48 h after direct plasma treatment. (f,g) Length (f) and length-to-width-ratio (g) of 4-dpf larvae after direct plasma treatment. (h) Representative images of dorsal and lateral views of zebrafish larvae after medium-covered plasma treatment. Scale bar = 2 mm (h). Data are mean ± SEM derived from three independent experiments ($n = 15$ eggs per condition). Significance was determined by one-way analysis of variance with Tukey's posttest for multiple comparisons or a two-tailed t test. *$p < .05$, **$p < .01$, and ***$p < .001$

generated via plasma systems, also to promote immunostimulation.[45–47] Next-generation physical plasma devices with various electric and gas combinations are being developed to target the tumor cells more efficiently. For instance, others and we have previously reported differential antitumor efficacies of different ROS patterns in vitro, usually generated by modulating the gas fed into plasma jets.[48–51] Moreover, we were able to report the relevance of optimizing the plasma-derived ROS composition in limiting syngeneic melanoma growth in vivo.[52] Yet, from an economical, practical, and ethical perspective, animal models are limited for screening many different plasma modes or sources in vivo. Hence, there is a need for a versatile and rapid in vivo model system to determine the biological and antitumor efficacy of evolving cold plasma devices. Albeit feed gas modulation was not done in this current study, we have provided assay systems and models of how such an assessment could be performed in zebrafish larvae to optimize plasma devices for biomedical applications. This might be of particular interest in zebrafish larvae, as these embryos can be engrafted with human and mouse tumor cells,[53] and they provide several practical advantages due to their conserved response to oxidative stress.[54]

Previous studies have focused on the role of H_2O_2 in tissue homeostasis, indicating that zebrafish are exceptionally receptive to oxidant-mediated cell signaling.[55,56] By contrast, oxidative stress also can alter embryonic development, both positively and negatively.[57] Several studies have investigated specific ROS in early zebrafish development, either using chemical or genetically encoded ROS sensors.[21,58] In the field of plasma medicine, it was reported that the treatment of extracellular matrix with a dielectric barrier discharge plasma system promoted chondrogenesis and bone formation in mice.[59] Another work investigating plasma treatment of chicken embryos suggested shorter plasma treatment times to promote their development, whereas longer treatment times abolished the Nrf2-related antioxidant defense, resulting in deformation and death.[60] This suggests hormetic effects of ROS, an observation made in redox biology already.[61,62]

In general, oxidants are known to disrupt the normal developmental processes of zebrafish embryos, leading to gross morphological defects.[63] We here report a subtle, but significant, change in morphology in some zebrafish larvae hatching from plasma-treated eggs. This may point to sublethal alterations in embryonal development, which may not only be related to plasma-derived ROS but also other plasma components present during direct treatment such as electric fields and UV radiation[64] that could potentially disrupt embryonic development. Nevertheless, the embryos exposed to direct plasma treatment had a mild reduction in the hatching rate and length-to-width-ratio, and most of the hatched larvae survived at 5 dpf. The observed morphological defects (failure in hatching, spinal deformity, and reduced pigmentation) were only seen at more extended treatment conditions. Regardless, it would be interesting to monitor the effect of nontoxic plasma treatment on the overall development of adult zebrafish. A previous study using another plasma jet found no differences in hatching rates and viability of plasma-treated larvae or changed vasculogenesis and regeneration of cut fins in plasma-treated adult fish.[65] Moreover, it was found that plasma treatment might stimulate neural growth in zebrafish,[66] making this model also interesting to study neurodegenerative diseases. In general, it should be mentioned that the kINPen plasma jet treatment is void of inducing mutagenic effects in vitro[67,68] and in ovo[69] as well as lacking long-term side effects or carcinogenic promotion in mice[70] and volunteers one year after plasma treatment.[71]

Overall, our results suggest the zebrafish larvae model to be a suitable future tool for plasma medicine studies. Further research is warranted to employ these findings in validating novel cold plasma devices and possibly monitoring the progress of orthotropic tumors in zebrafish embryos in cancer research.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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