Lipidomics of Microplasma-Irradiated Cells at Optimized Discharge Conditions for the Absorption of High-Molecule Drug

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Abstract: Microplasma irradiation is a promising technique for the transdermal delivery of high-molecular-weight drugs. In this technique, microplasma components interact with the skin surface or cell membranes, allowing the drugs to penetrate. For efficient and safe drug delivery, it is crucial to understand these interactions. To this end, this study investigated the effects of microplasma irradiation on cellular lipids, particularly those associated with cell membranes. Rat intestinal epithelial cells were treated with microplasma irradiation at two different voltages (4.0 kV or 4.5 kV). An untargeted lipidomic was conducted using liquid chromatography–mass spectrometry (LC/MS) technique. The results revealed that microplasma irradiation at 4.0 kV induces a significant increase in cell membrane lipids within 10 min post-irradiation. All major cell membrane lipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin, exhibited increases of over 25% within this short timeframe. Notably, this effect is transient, as lipid levels return to their baseline after 12 h. Furthermore, no significant differences in live and apoptotic cell percentages were observed between the control and 12 h post-irradiated cells. In contrast, irradiation at 4.5 kV did not elicit significant changes in cell membrane lipids, correlating with the absence of drug absorption under this condition. Hence, our study unveiled a correlation between the rapid increase in cell membrane lipids and enhanced drug absorption in microplasma-irradiated cells. This lipid augmentation potentially enhances membrane fluidity and permeability, thus facilitating drug absorption. Beyond elucidating the mechanisms and safety of microplasma-based drug delivery, our research provides valuable insights for advancing various microplasma-based biomedical technologies.

Keywords: cell membrane; drug delivery; lipid; microplasma

1. Introduction

Microplasma, a form of cold atmospheric plasma (CAP) occurring on a microscale dimension [1,2], represents a rich source of active species and elements, including reactive oxygen species (ROS), reactive nitrogen species (RNS), ions, excited atoms and molecules, electrons, and electromagnetic radiation [3]. The medical applications of CAP,
particularly in dermatology, are well-known [4]. Recently, microplasma irradiation has attracted attention for its potential to enhance transdermal drug delivery and cellular drug absorption [5]. In 2017, our group demonstrated the transdermal delivery of a low-molecular-weight drug, cyclosporine A (molecular weight of 1203 Da), using a CAP jet or microplasma irradiation [6].

In conventional drug delivery systems, including oral, parenteral (such as intravenous and intramuscular), inhalation, and transdermal delivery, each approach has its own advantages and limitations. Oral delivery remains the most convenient and widely used method. However, this route faces several challenges, including the first-pass metabolism in the liver, enzymatic degradation, pH variation in the gastrointestinal tract, and potential interactions with food, which can reduce the bioavailability of drugs. Parenteral delivery ensures rapid onset of action and precise dosing, but is often associated with discomfort, pain, and tissue damage at the administration site. Additionally, parenteral administration requires trained healthcare professionals and carries a risk of infection. Inhalation delivery proves effective for treating respiratory conditions, but it faces challenges such as the need for proper coordination during inhalation, device maintenance, and the potential risk of lung irritation or damage. In contrast, transdermal drug delivery offers several advantages, including targeted delivery, painlessness, reduced drug doses and toxicity, avoidance of first-pass metabolism in the liver, and improved drug efficacy and stability. However, certain drugs, especially those with high molecular weights exceeding 500 Daltons, face limitations in transdermal delivery under normal circumstances. In order to address this challenge, microplasma irradiation emerges as a promising approach to enhance the transdermal delivery of drugs.

The plasma treatment of tissue can be painful depending on the type of plasma device used. Some plasma can cause holes in the stratum corneum, and this treatment can be potentially painful, as reported in the study of Gelker et al. [7]. On the other hand, microplasma used in the drug delivery is painless. This type of plasma can cause etching of the most upper part of the skin—the stratum corneum—up to several micrometers [8].

Intestinal epithelial cells are often used in microplasma-based drug delivery studies as they offer greater permeability than skin. Drug permeation through the intercellular pathway of the skin is limited to 500 Da, while intestinal tissue allows permeability of molecules with molecular weights of 70 kDa without any enhancers [9,10]. Recently, we showed the capability of microplasma irradiation to deliver high-weight molecules with molecular weights of up to 2000 kDa into rat intestinal epithelial cells [11,12].

The interactions between CAP and skin or cells are largely unexplored. It has been shown that the reactive species can form nanopores in artificial cell membranes [13]. During the transdermal application of plasma, the ROS and RNS can penetrate 150 µm–1.5 mm under the skin’s surface [14], suggesting interactions with both superficial (i.e., the stratum corneum layer) and deeper skin layers (i.e., epidermal and dermal cells).

Cell membranes of both humans and animals mainly comprise diverse lipid species, arranged in a lipid bilayer [15] that plays a crucial role in determining the permeability of the membranes [16]. Microplasma irradiation has the potential to induce changes in the lipid composition of the cell membrane, affecting its fluidity, permeability, and overall structural integrity. Understanding the changes in these properties is crucial for evaluating the safety and long-term effects of plasma technology in biomedical applications, including transdermal drug delivery.

In a recent study, we optimized the microplasma irradiation conditions for the delivery of high-molecular-weight molecules, fluorescein isothiocyanate (FD-150 and FD-2000), into rat intestinal epithelial cells. Further, we investigated a crucial physical characteristic of cell membranes, the membrane lipid order, after microplasma treatment at the optimized conditions. While the untreated cells exhibited an exclusive liquid-ordered phase, the microplasma-irradiated cells displayed both liquid-ordered and liquid-disordered phases [12]. The study suggested that the altered membrane lipid order facilitates the transport of molecules through the cell membrane by modulating the fluidity and permeability.
of the membrane. These findings led us to conduct comprehensive lipidomics of cells after microplasma irradiation in order to identify the specific lipid species that contribute to membrane fluidity and permeability, shedding light on the dynamic nature of the cell membrane in response to microplasma irradiation.

2. Materials and Methods

2.1. Preparation of Cell Culture Medium

We purchased the powder of Dulbecco’s Modified Eagle Medium (DMEM) containing high glucose and phenol red from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). The antibiotic solution (penicillin–streptomycin), L-glutamine solution, powder of sodium bicarbonate, 0.25% (w/v) Trypsin-1mM EDTA.4Na with phenol red, and recombinant insulin (human source) solution were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Fetal bovine serum (FBS) originating from Australia was purchased from Serana Europe GmbH (Pessin, Germany).

In order to prepare the cell culture medium, the required ingredients were mixed as follows: (i) 4.25 g of DMEM powder, (ii) 500 mL of distilled water, (iii) 25 mL of FBS, (iv) L-glutamine solution (5 mL), (v) 5 mL of antibiotic solution, (vi) 200 µL of insulin, and (vii) 10% sodium bicarbonate solution (8 mL).

2.2. Cell Culture in Flasks

The small intestine epithelial cells (IEC6) of rats were obtained from the Riken Cell Bank (Tsukuba, Japan). Initially, culture medium (3 mL) was taken in a 25 cm$^2$ culture flask. The cells were seeded into the culture medium, followed by incubation at 37 °C with a 5% CO$_2$ environment. The cells attached to the bottom layer of the flask within one day. The culture medium was replaced with fresh medium every day.

2.3. Transfer of Cells from the Flask to Culture Dishes

The cells in the flask became confluent in 2–3 days. The confluent cells were then transferred to culture dishes (Figure 1). Before that, the culture medium was gently aspirated from the culture flask, followed by washing (twice) with phosphate-buffered saline (PBS). For the detachment of the cells, 1 mL of trypsin solution was gently poured onto the surface of the culture flask and half of the solution was immediately removed. In addition, tapping on the flask’s walls was done in order to help in the efficient detachment of the cells. After that, an appropriate volume of culture medium was added to the detached cells and mixed well. The resulting suspension was transferred to the required number of 60 mm × 15 mm culture dishes (3 mL/dish), followed by incubation at 37 °C with 5% CO$_2$.

2.4. Microplasma Treatment

The applied microplasma irradiation conditions were based on our published literature [12]. IEC6 cells cultured in dishes (60 mm × 15 mm) were irradiated with microplasma discharge for a duration of 2 min. A thin-film electrode was used to generate a dielectric barrier discharge under atmospheric conditions. The microplasma irradiation did not use any gas flow. The distance between the surface of the cell medium and the thin-film electrode was approximately 2 mm. Two voltage parameters (i.e., 4 kV or 4.5 kV), at a frequency of 5 kHz, were used for the treatment. A function generator (AFG3102, Tektronix, Beaverton, OR, USA) was used to set a triangle (positive and negative) waveform, which was subsequently amplified by a high-voltage amplifier (5/80, Trek, New York, NY, USA). The time-point of microplasma irradiation to different group samples is shown in Figure 1.
Figure 1. Workflow of cell culture and microplasma treatment for LC/MS and flow cytometry analyses. (a) Set A and (b) Set B samples were used for LC/MS analysis. In both cases, cells were initially cultured in flasks for 2–3 days followed by transfer to culture dishes. In Set A, samples were divided into three groups: (i) no irradiation (control), (ii) microplasma irradiation at 4.0 kV followed by 0 h incubation (A4K0H), and (iii) microplasma irradiation at 4.0 kV followed by 1 h incubation (A4K1H). In Set B, samples were divided into four groups: (i) no irradiation (control), (ii) microplasma irradiation at 4.0 kV followed by 0 h incubation (B4K0H), (iii) microplasma irradiation at 4.0 kV followed by 12 h incubation (B4K12H), and (iv) microplasma irradiation at 4.5 kV followed by 0 h incubation (B4.5K0H). The shaded boxes indicate the timing of microplasma irradiation for the respective samples. Samples were prepared to ensure that the total incubation time for each sample within a particular set remained consistent (with variations of ±0.25 h). Cells were washed and snap-frozen within 10 min after microplasma treatment or after an additional incubation period (if any) after irradiation. (c) Set C samples were prepared similarly to Set B samples and were utilized for flow cytometry analysis. Sample preparation (from cell culture to LC/MS analysis/flow cytometry analysis) of individual sets were carried out independently.
2.5. Lipid Extraction from Cells

Ultra-pure water, pure chloroform (HPLC grade), and pure methanol (LC/MS grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

The cells were detached from the surface of the culture dish using a cell scrapper and transferred into a glass tube (Iwaki, Tokyo, Japan). The dish was further washed with 3 mL of PBS in order to collect any remaining cells. The glass tube was subjected to centrifugation at 2000 rpm (2 min) in order to pellet the cells at the bottom of the tube. The supernatants were removed from the tube. PBS (3 mL) was added to the glass tube, followed by centrifugation (2000 rpm, 2 min). The supernatant was discarded, the cells were snap-frozen using dry ice, and then immediately placed in a freezer at −80 °C. The following day, lipid extraction was carried out from the stored cells using the Bligh and Dyer method [17], with a slight modification. In order to initiate the extraction, 0.8 mL of ultra-pure water, 2 mL of pure methanol, and 1 mL of pure chloroform were added to each glass tube. The mixture was subjected to sonication for 10 min and then left at room temperature for half an hour. After that, the appropriate amount of chloroform (1 mL) was added to the tube and mixed well. Finally, 1 mL of ultra-pure water was added to the tube and mixed well again. The extract was left for 10 min in order to allow for phase separation. After that, all of the sample tubes were centrifuged at 3000 rpm (10 min) in order to obtain the clear chloroform layer. An equal volume (1 mL from each tube) of the lower phase (chloroform layer) was transferred to another glass tube. Chloroform was placed in a rotary evaporator, and the dry lipid extract was stored in a freezer at −80 °C for one day.

2.6. LC/MS Analysis

The LC/MS method employed in this study was based on a reported study [18]. The dry lipids were reconstituted with pure methanol. In order to remove any particles, the sample solution was filtered (filter pore size: 0.20 µm) and then transferred into an LC/MS insert vial. The LC/MS analysis was conducted employing a highly sensitive Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) attached to an ultra-high performance liquid chromatography unit (Ultimate 3000 system, Thermo Fisher Scientific). Ions of the lipid species were generated using an electrospray ionization (ESI) source.

A 5 µL sample was injected by an autosampler, and the lipids were separated in a hydrophobic column (Acclaim 120 C18, 150 mm × 2.1 mm, 3 µm, Thermo Scientific, Waltham, MA, USA). Throughout the analysis, the LC column and the autosampler chamber were kept at 50 °C and 10 °C, respectively. The mobile phase (A and B) was delivered at a flow rate of 300 µL/min for elution. Mobile phase A was prepared as follows: water–acetonitrile–methanol (2:1:1 v/v/v), 0.1% formic acid, and 5 mM ammonium formate. Mobile phase B consisted of acetonitrile–isopropanol (1:9 v/v), 0.1% formic acid, and 5 mM ammonium formate. The elution conditions were programmed as follows: gradients starting at 20% B, linearly increased to 100% B in 50 min, and isocratic at 100% B for 10 min. At 60.1 min, the gradient returned to the initial condition (20% B) and was maintained at 20% B until 70 min in order to equilibrate the column. Blank (pure methanol) was run before each sample injection.

The MS conditions were optimized as follows: capillary temperature, 250 °C; probe heater temperature, 350 °C; spray voltage, 3.5 kV (for positive mode) and 2.5 kV (for negative mode); S-lens RF level, 50; auxiliary gas flow, 15 (au); sweep gas flow, 0 (au); and sheath gas flow, 50 (au). Data were acquired in both full MS and data-dependent MS/MS (dd-MS2) modes using Xcalibur v3.0 Software (Thermo Scientific). The settings for full MS mode were as follows: automatic gain control (AGC) target, 1 × 10⁶; m/z range, 220–2000; mass resolving power, 70,000 (FWHM, at m/z 200); and maximum sample injection time (IT), 100 ms. The following conditions were used for dd-MS2: mass resolving power of 17,500 (FWHM, at m/z 200); AGC target of 1 × 10⁵; maximum IT of 80 ms; loop count of 5; TopN of 5; isolation window of 2.0 m/z; normalized collision energy (NCE) of 30.0 eV;
dynamic exclusion of 15.0 s; and stepped NCE of 15.0% for positive mode and 35.0% for negative mode.

The raw data were directly subjected to LipidSearch™ software version 4.2.13 (Mitsui Knowledge Industry, Tokyo, Japan) for lipid annotation and semi-quantification. The parameter settings are provided in Table S1. The processed data were exported to MS Excel (2021) in order to perform statistical analysis and to create necessary charts or plots.

2.7. Flow Cytometry Analysis

For studying early apoptosis, apoptosis, and necrosis, a third set of samples was prepared (Figure 1). Cell culture, microplasma treatment, and incubation conditions were similar to that of the set 2 samples. The cells were trypsinized, transferred to a centrifuge tube, and then washed with PBS. Next, \(1 \times\) binding buffer (100 \(\mu\)L), FITC annexin V (5 \(\mu\)L), and propidium iodide (5 \(\mu\)L) were added and mixed by gentle pipetting. After that, the samples were incubated at room temperature for 15 min. After the incubation, an extra amount of \(1 \times\) binding buffer (400 \(\mu\)L) was added again to each tube. Non-irradiated cells with staining (FITC annexin V and propidium iodide) or without staining were taken as the control. Flow cytometry analysis was performed by BD Accuri™ C6 Plus Flow Cytometer, (BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer’s recommended protocol.

2.8. Statistical Analysis

For the statistical analysis, Student \(t\)-tests were performed using MS Excel and considered significant at \(p < 0.05\).

3. Results and Discussion

We performed untargeted lipidomics of microplasma-irradiated cells in order to investigate the effects of microplasma irradiation on cellular lipids, particularly cell membrane lipids. The microplasma irradiation conditions employed in this study were based on our previous research, wherein a voltage of 4.0 kV was identified as optimal for facilitating the absorption of larger molecules into cells [12].

In this study, two independent sets of samples, labeled as set A and set B, were subjected to LC/MS analysis (Figure 1). Set A consisted of three groups of cell samples: (i) non-irradiated (control); (ii) subjected to microplasma irradiation at 4.0 kV, followed by 0 h of incubation (A4K0H); and (iii) microplasma irradiation at 4.0 kV, followed by 1 h of further incubation (A4K1H). On the other hand, set B samples were divided into four groups: (i) non-irradiated (control); (ii) microplasma irradiation at 4.0 kV, followed by 0 h of incubation (B4K0H); (iii) microplasma irradiation at 4.0 kV, followed by 12 h of further incubation (B4K12H); and (iv) microplasma irradiation at 4.5 kV, followed by 0 h of incubation (B4.5K0H).

According to the LIPID MAPS (http://www.lipidmaps.org/, accessed on 2 May 2024), lipids have been divided into eight categories as follows: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PL), saccharolipids, and polyketides [19]. In this study, we used LipidSearch software to annotate and quantify all available lipid classes detected in the control and treated cells. After filtering the annotations (parameters for filtration are listed in Table S1), a total of 1724 and 1577 lipid species were identified in set A and set B samples, respectively (Table 1). These lipids belong to five categories (GL, GP, SP, ST, and PL) and twenty-one classes. The four most abundant lipid classes (in terms of the number of individual species) detected in both sets of samples were phosphatidylcholine (PC), phosphatidylethanolamine (PE), triglyceride (TG), and ceramide (Cer) (Table 1).
Table 1. Lipid categories and classes detected in set A and set B cell samples.

<table>
<thead>
<tr>
<th>Lipid Categories</th>
<th>Lipid Class</th>
<th>Number of Species Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Set A</td>
</tr>
<tr>
<td>Glycerolipids (GL)</td>
<td>Monoglycerides (MG)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Diglycerides (DG)</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Triglycerides (TG)</td>
<td>335</td>
</tr>
<tr>
<td>Glycerophospholipids (GP)</td>
<td>Phosphatidic acid (PA)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylcholine (PC)</td>
<td>461</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylethanolamine (PE)</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylserine (PS)</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylinositol (PI)</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylglycerol (PG)</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Lysocephatidylcholine (LPC)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Lysocephatidylethanolamine (LPE)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Lysocephatidylinositol (LPI)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cardiolipin (CL)</td>
<td>16</td>
</tr>
<tr>
<td>Sphingolipids (SP)</td>
<td>Ceramide (Cer)</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>Sphingomyelin (SM)</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Monohexosylceramide (Hex1Cer)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Dihexosylceramide (Hex2Cer)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Sphingosine (SPH)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Gangliosides (GM3)</td>
<td>4</td>
</tr>
<tr>
<td>Sterol lipids (ST)</td>
<td>Cholesteryl ester (CE)</td>
<td>35</td>
</tr>
<tr>
<td>Prenol lipids (PL)</td>
<td>Coenzyme Q (CoQ)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Total number of lipid species</td>
<td>1724</td>
</tr>
</tbody>
</table>

3.1. Changes in Lipids Associated with Cell Membranes

We first focused on investigating the changes in lipids associated with the cell membrane. Four classes of lipids predominate in the cell membrane of many mammalian cells: PC, PE, phosphatidylserine (PS), and sphingomyelin (SM). PC, PE, and PS fall under the category of GP, while SM is classified as SP. Other lipids contributing to cell membrane formation include hexosylceramide and ganglioside, both belonging to the SP category, along with cholesterol [20]. In our study, all of the cell membrane-associated GP were detected in both sets of samples. Of the SP category, SM, GM3, monohexosylceramide (Hex1Cer) and dihexosylceramide (Hex2Cer) were detected (Tables 2 and 3). However, cholesterol was not detected. This could be due to the fact that cholesterol abundance in epithelial cells is low and it shows poor ionization efficiency under electrospray ionization conditions [21].

In our study, it was found that PC constituted approximately 80% of all GP comprising the cell membrane, with PE, PS, and PI following in decreasing order of abundance (Tables 2 and 3). Our results are consistent with those previously published. It has been evidenced by numerous studies that PC is the most abundant and PE is the second most abundant GP in all mammalian cells [22]. Additionally, SM comprised roughly 5–7% of the total cell membrane lipids and was the most abundant species within the SP category (Tables 2 and 3).
Table 2. Composition of cell membrane lipids in set A cell samples.

<table>
<thead>
<tr>
<th>Lipid Categories</th>
<th>Classes</th>
<th>Composition (%) (Calculated from Mean Area Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>Glycerophospholipid (GP)</strong></td>
<td>Phosphatidylcholine (PC)</td>
<td>82.76</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylethanolamine (PE)</td>
<td>9.70</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylserine (PS)</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylinositol (PI)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Sphingolipid</strong></td>
<td>Sphingomyelin (SM)</td>
<td>4.84</td>
</tr>
<tr>
<td></td>
<td>Ganglioside (GM3)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Monohexosylceramide (Hex1Cer)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Dihexosylceramide (Hex2Cer)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Sum</strong></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3. Composition of cell membrane lipids in set B samples.

<table>
<thead>
<tr>
<th>Lipid Categories</th>
<th>Classes</th>
<th>Composition (%) (Calculated from Mean Area Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>Glycerophospholipids (GP)</strong></td>
<td>Phosphatidylcholine (PC)</td>
<td>79.73</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylethanolamine (PE)</td>
<td>10.29</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylserine (PS)</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylinositol (PI)</td>
<td>0.90</td>
</tr>
<tr>
<td><strong>Sphingolipids (SP)</strong></td>
<td>Sphingomyelin (SM)</td>
<td>7.57</td>
</tr>
<tr>
<td></td>
<td>Ganglioside (GM3)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Monohexosylceramide (Hex1Cer)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Dihexosylceramide (Hex2Cer)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Sum</strong></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Following microplasma irradiation, there was a significant ($p < 0.05$) increase in total cell membrane lipids within 10 min (A4K0H cells) compared to the control (Figure 2a). Initially, we hypothesized that with further incubation at 37 °C (with 5% CO$_2$) for 1 h post-irradiation, the lipid levels would revert to those of the control group. Contrary to our expectations, even after 1 h of incubation (A4K1H cells), the total cell membrane lipids remained significantly elevated. Notably, among the GP category, PS levels showed the highest increase, approximately 50%, and this elevation persisted during the post-irradiation incubation. PC, PE and PI levels showed similar patterns—they increased in A4K0H cells and then started to decrease in A4K1H cells (Figure 2c). The changes in PC and PE levels were very similar, both experiencing approximately 30% increases in A4K0H cells (Figure 2c). Two SP classes, SM and Hex1Cer, were increased by approximately 40% in A4K0H cells compared to the control. While SM levels continued to rise sharply in A4K1H cells, Hex1Cer levels remained relatively stable (Figure 2d). Given the inconsistency between our hypothesis and the observed lipid changes in A4K1H cells, we conducted a secondary analysis involving samples collected up to 12 h post-irradiation (B4K12H cells). Consistent with our initial findings (i.e., the results of set A), total cell membrane lipids were significantly ($p < 0.05$) elevated in B4K0H cells compared to the controls in set B. As anticipated, there were no significant differences in lipid levels between B4K12H cells and the controls, indicating a return to normal levels 12 h post-irradiation (Figure 3a,b).
cells, Hex1Cer levels remained relatively stable (Figure 2d). Given the inconsistency between our hypothesis and the observed lipid changes in A4K1H cells, we conducted a secondary analysis involving samples collected up to 12 h post-irradiation (B4K12H cells). Consistent with our initial findings (i.e., the results of set A), total cell membrane lipids were significantly ($p < 0.05$) elevated in B4K0H cells compared to the controls in set B. As anticipated, there were no significant differences in lipid levels between B4K12H cells and the controls, indicating a return to normal levels 12 h post-irradiation (Figure 3a,b).

Figure 2. Changes in cell membrane lipids until 1 h following microplasma irradiation at 4.0 kV condition. These data correspond to set #1 samples. (a) Total cell membrane-associated lipid alterations were determined from the absolute area values derived from LC/MS analysis. Statistical analysis compared the A4K0H group (n = 5) and A4K1H (n = 5) groups with the control group (n = 5). (b) Percentage changes in total cell membrane-associated lipids in the A4K0H and A4K1H groups relative to the control. (c) Relative changes in glycerophospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) species in A4K0H and A4K1H groups compared to the control. (d) Relative changes in sphingomyelin (SM), ganglioside (GM3), monohexosylceramide (Hex1Cer), and dihexosylceramide (Hex2Cer) species in the A4K0H and A4K1H groups compared to the control.
sphingomyelin (SM), ganglioside (GM3), monohexosylceramide (Hex1Cer), and dihexosylceramide (Hex2Cer) species in the A4K0H and A4K1H groups compared to the control. The trends observed among the GP classes were remarkably uniform: they exhibited a consistent elevation in B4K0H cells, followed by a restoration to baseline levels (i.e., almost similar to the control cells) in B4K12H cells (Figure 3c). On the other hand, SP classes showed variability in their changes (Figure 3d).

Figure 3. Changes in cell membrane lipids until 12 h after microplasma irradiation at 4.0 kV condition. These data belong to set #2 samples. (a) Total cell membrane-associated lipid alterations were calculated based on the absolute area values obtained from LC/MS analysis. Statistical analysis compared the B4K0H group (n = 5) and B4K12H group (n = 5) with the control group (n = 5). (b) Relative changes (expressed in percentages) in total cell membrane-associated lipids in the B4K0H and B4K12H groups compared to the control. (c) Relative changes in phosphatidylcholines (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) species in the B4K0H and B4K12H groups compared to the control. (d) Relative changes in sphingomyelin (SM), ganglioside (GM3), monohexosylceramide (Hex1Cer), and dihexosylceramide (Hex2Cer) species in the B4K0H and B4K12H groups compared to the control.
The trends observed among the GP classes were remarkably uniform: they exhibited a consistent elevation in B4K0H cells, followed by a restoration to baseline levels (i.e., almost similar to the control cells) in B4K12H cells (Figure 3c). On the other hand, SP classes showed variability in their changes (Figure 3d).

The absorption of drugs into cells was markedly enhanced during or immediately following microplasma irradiation, as suggested by previous research [11]. Notably, there was a significant increase in cell membrane lipids within 10 min post-irradiation, suggesting a direct correlation between lipid concentrations and drug uptake. It has been reported that lipids play a crucial role in determining the permeability of cell membranes [16], which supports the hypothesis that lipid augmentation may facilitate drug absorption by altering membrane characteristics. It would be more interesting if the lipid changes were studied immediately after the irradiation. However, the inherent nature of the LC/MS sample preparation renders such timely studies challenging, limiting our understanding of these immediate biochemical changes.

The increase in lipids observed in A4K0H or B4K0H cells cannot be attributed to a potential increase in cell numbers after irradiation. Rat epithelial cells typically undergo a cell cycle spanning a day or longer [23], rendering substantial proliferation unlikely within the short timeframe of our experimental protocol. Moreover, the A4K0H and B4K0H cells were washed and snap-frozen within 10 min of irradiation, which excludes the possibility that increased lipid levels originated from cellular proliferation. Hence, it is reasonable to hypothesize that microplasma irradiation stimulated lipid synthesis within the cells, leading to an augmentation in lipid content—a phenomenon often associated with lipogenesis.

It was expected that we would observe oxidized lipid species in microplasma-irradiated cells, since ROS species are known to cause lipid peroxidation [24]. However, we could not find any oxidized lipid species in the irradiated cells. This could be due to the analytical challenges, such as the low abundance, poor stability, the limited ionization efficiency of oxidized lipids [25,26], and the limitations of lipid annotation tools. Our findings are consistent with a previous study, wherein the oxidative modification of skin lipids by CAP was investigated by MS/MS, but oxidized lipid species was rarely detected [27]. Polyunsaturated fatty acids (PUFAs) are highly susceptible to peroxidation due to the presence of multiple carbon–carbon double bonds [28]. Therefore, we analyzed PUFA-containing PC species in order to investigate the lipid peroxidation indirectly. However, our analysis revealed a significant increase in total PUFA-containing PC species in B4K0H cells (Figure S1), indicating that peroxidation was not a major phenomenon in the irradiated cells. These findings resonate with a recent study by Li et al., who observed a notable increase in PUFA chains within kidney PC, PE, and TG species within 5 min of whole-body irradiation with 10 Gy and 20 Gy in mice [29].

One of the limitations of this study is that the lipids were extracted from whole cells, meaning that the cell membrane-associated lipids mentioned above did not exclusively reflect the composition of the cell membranes. Eukaryotic cells harbor many membrane-bound organelles and lipid droplets, which share major lipids with cell membranes [30,31]. Obtaining lipids from isolated cell membranes would offer more precise insights into the dynamics of microplasma-irradiated cell membranes. However, a lipidomic analysis of isolated cell membranes presents challenges due to the complexity and inefficiency of sample preparation procedures.

3.2. Changes in Lipids Other than Cell Membrane Lipids

Next, we analyzed lipids not directly involved with cell membrane structures. Such lipids detected in our study were PG, PA, lyso-glycerophospholipids (Lyso-GP), GL, CE, Cer, SPH, CL, and CoQ. Notably, the collective pool of non-cell membrane lipids exhibited a significant increase in B4K0H cells compared to the control group. Interestingly, in contrast to cell membrane lipids, these lipids showed a further elevation in B4K12H cells compared to B4K0H cells (Figure 4a).
Figure 4. Alterations in non-cell membrane lipids until 12 h following microplasma irradiation at 4.0 kV condition. Data were obtained from set #2 samples. (a) Changes in total non-cell membrane lipids were determined based on the absolute area values derived from LC/MS analysis. Statistical analysis compared B4K0H group (n = 5) and B4K12H group (n = 5) with the control group (n = 5). (b–f) Relative changes (expressed in percentages) in various non-cell membrane lipids in B4K0H and B4K12H groups were compared to that of the control group.

PG is a minor component of many subcellular membranes, particularly mitochondrial membranes [32]. PA is a precursor of other GP species and acts as a lipid second messenger in extracellular and intracellular signaling [33]. In our study, PG and PA showed an increase of approximately 32% and 81% in B4K0H cells, respectively, compared to the control cells. In B4K12H cells, PG levels were comparable to the control cells, while PA levels saw a significant decrease (Figure 4b).
All lyso-GP classes experienced increases in B4K0H cells, with LPI showing the highest increase at around 66% (Figure 4c). Similarly, all GL and CE species showed increases in both B4K0H and B4K12H cells compared to the control, with TG displaying the most prominent increase (Figure 4d). It is worth noting that lyso-GP are intercellular signaling molecules [34], while TG and CE serve as major storage lipids in cells [35].

SPH decreased by approximately 18% in B4K0H cells, but increased by 18% in B4K12H cells compared to the control (Figure 4e). CL, a mitochondrial unique lipid, exhibited a 55% increase in B4K0H cells and then become 114% in B4K12H cells compared to the control cells (Figure 4f). CoQ, an enzyme co-factor in the mitochondrial respiratory chain, followed a similar pattern to CL. These results suggest that microplasma components not only interacted with the cell membrane, but also penetrated into the cells. Although the intracellular lipid levels increased immediately after irradiation, most of them (except GL and CE) showed a tendency to return to a normal level after 12 h of irradiation.

3.3. Changes in Lipids in Cells Irradiated at 4.5 kV

In our previous study, we observed a significant reduction in cell viability following microplasma irradiation at 4.5 kV, with an additional 1 h incubation period. Also, we did not find any differences in the drug absorption into cells between the control cells and those irradiated with microplasma at 4.5 kV [12]. In this current study, we investigated the changes in lipids at this specific irradiation condition. The cells were subjected to microplasma irradiation at 4.5 kV and then processed for lipid extraction within 10 min post-irradiation (referred to as B4.5K0H). Our analysis revealed no significant differences in both total cell membrane lipids and total non-membrane lipids between the B4.5K0H and control cell groups (Figure 5). Hence, these findings clearly indicate that drug absorption is directly associated with the changes in cell membrane lipids.

3.4. Analysis of Cell Death after Microplasma Treatment

A flow cytometry analysis was conducted on set C samples (Figure 1) in order to examine the potential relationship between lipid alterations and cell death. Cell death can occur in two forms: accidental cell death (i.e., necrosis) and regulated cell death (e.g., apoptosis, ferroptosis, autophagy, etc.) [36]. We employed an Annexin V binding assay, which enabled the discrimination of live cells, early apoptotic cells, late apoptotic cells, and necrotic cells in both control and microplasma-irradiated cells.

Annexin V is a calcium-dependent protein that specifically binds PS on the outer leaflet of the cell membrane. In healthy cells, PS is normally found in the inner leaflet (cytoplasmic site) of the cell membranes. However, during apoptosis, PS translocates to the outer leaflet of the cell membrane. This translocation of PS is detected through FITC conjugated Annexin V. Propidium iodide is often used along with Annexin V for studying apoptosis. It selectively labels nucleic acids of dead and late apoptotic cells. Single positive for Annexin V staining indicates apoptotic cells, while double-positive staining for Annexin V and propidium iodide marks necrotic cells [37].

Our results revealed that microplasma irradiation at 4.0 kV without further incubation (designated as C4K0H cells) led to a significant increase in live cells ($p = 0.002$), accompanied by a significant decrease in apoptotic ($p = 0.005$) and necrotic cells ($p = 0.0002$) compared to the control samples (Figure 6). The notable cell death observed in the control samples was likely due to the inherent factors of the staining procedure, which included trypsinization, washing, centrifugation, and exposure to staining reagents at room temperature. Thus, microplasma irradiation appears to exert a protective effect against apoptosis and necrosis in C4K0H cells, potentially mediated by short-lived species generated in the aqueous medium by the microplasma [38].
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Figure 5. Changes in lipids within 10 min following microplasma irradiation at 4.5 kV condition. (a) Changes in total cell membrane-associated lipids, and (b) total non-cell membrane lipids were calculated based on the absolute area values obtained from LC/MS analysis (set #2 data). Statistical analysis was performed to compare the B4.5K0H group (n = 5) with the control group (n = 5). (b) Relative changes (expressed in percentages) in (c) glycerophospholipids and (d) sphingolipids in the B4.5K0H group were compared to those in the control group.

On the other hand, microplasma irradiation at 4.0 kV followed by 12 h incubation (C4K12H cells) did not exhibit significant differences in live cells and apoptotic cells compared to the controls. However, early apoptosis was significantly (p = 0.0006) increased, while necrosis was significantly (p = 0.0002) decreased in C4K12H cells relative to the controls. These findings suggest that the effects of microplasma irradiation no longer exist 12 h after the treatment.

Furthermore, irradiation at 4.5 kV with no subsequent incubation (C4.5K0H cells) significantly increased live cells (p = 0.004) and reduced apoptotic (p = 0.009) and necrotic cells (p = 0.0002). Nonetheless, compared to C4K0H cells, live cells in C4.5K0H were diminished, while early and late apoptotic cells, as well as necrotic cells, were approximately twofold higher. These data support that the 4.0 kV condition is more favorable for cell viability than the 4.5 kV condition.

It is worth noting that cell death by irradiation is not limited to apoptosis and necrosis. Cells may undergo other forms of regulated cell death, including ferroptosis [39,40], which was not explored in this study.
Figure 6. Analysis of cell death in microplasma-irradiated cells using flow cytometry. (A) Representative dot plots illustrating the relative counts of live, early apoptotic, apoptotic, and necrotic cells in both control and microplasma-irradiated cell populations. (B) Bar charts show the proportions of live, early apoptotic, apoptotic, and necrotic cells in microplasma-irradiated cells and control cells. The data are presented as mean ± standard deviation (n = 3). Statistical analysis compared the C4K0H group (n = 3), C4K12H group (n = 3), and C4.5K0H groups with the control group (n = 3). UL: necrotic cells; UR: late apoptotic cells; LL: viable cells; LR: early apoptotic cells.

4. Conclusions

This study conducted a comprehensive lipidomics analysis of microplasma-irradiated cells, utilizing highly sensitive LC/MS techniques. Rat intestinal epithelial cells were
subjected to microplasma irradiation at 4.0 kV, a condition previously identified as optimal for facilitating the absorption of high-molecular-weight drugs (up to 2000 kDa) into cells. Additionally, cells were irradiated at 4.5 kV in order to investigate lipid changes under extreme conditions.

The results demonstrate that irradiation at 4.0 kV induces a significant increase in cell membrane lipids within 10 min post-irradiation. This lipid augmentation potentially enhances cell membrane permeability, facilitating the penetration of high-molecular-weight drugs. Notably, the levels of cell membrane lipids return to baseline levels 12 h after irradiation, indicating a transient effect of microplasma on the cell membrane.

Conversely, irradiation at 4.5 kV did not elicit significant changes in cell membrane lipids. Consistent with our previous findings where drug absorption was not observed under this condition, our current study unequivocally demonstrates that microplasma promotes drug absorption by modulating cell membrane lipids.

Flow cytometry analysis revealed no significant differences in live cell and apoptotic cell percentages between the control and 12 h post-irradiated groups (at 4.0 kV), aligning with the observations from the LC/MS analysis. This consistency between flow cytometry and LC/MS data underscores the reliability of our findings.

In summary, our study elucidates the transient modulation of cell membrane lipids by microplasma irradiation, thereby potentially facilitating drug absorption. These insights contribute to our understanding of the mechanisms underlying microplasma-mediated cellular responses and hold potential implications for drug delivery strategies as well as other microplasma-based biomedical technologies.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14103978/s1, Table S1: LipidSearch (version 4.2.13) software parameters. Figure S1: Changes in PUFA-PC after microplasma irradiation at 4.0 kV condition.


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References


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