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Antiviral activity of palmitic acid via autophagic flux inhibition in zebrafish (*Danio rerio*)

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ABSTRACT

Fatty acids (FAs) are key elements that affect not only growth but also different immune functions, and therefore, nutrition is important for growing healthy fish. Zebrafish (*Danio rerio*) is a good model for assessing the beneficial effects of immunostimulants, including FAs, before applying them in aquaculture. Accordingly, this study evaluated the effects of palmitic acid (PA) treatment on different immune parameters of zebrafish and on the mortality caused by the spring viremia of carp virus (SVCV). The results suggest that PA modulates the infection outcome *in vivo*, which benefits zebrafish and results in reduced mortality and viral titres. The antiviral protection elicited by this FA seems to be associated with the inhibition of autophagy and is independent of other immune processes, such as neutrophil proliferation or type I interferon (IFN) activity. The use of PA as an immunostimulant at low concentrations showed great potential in the prevention of SVCV infections; therefore, this FA could help to prevent the mortality and morbidity caused by viral agents in aquacultured fish. Nevertheless, the potentially detrimental effects of suppressing autophagy in the organism should be taken into account.

1. Introduction

Fatty acids (FAs) are involved in diverse biological processes, including the modulation of vascular resistance, growth, survival, stress, cell membrane structure and immune responses. As a consequence, they have a powerful impact on overall health status. In addition to their ability to alter the fluidity of immune cell membranes, FAs also have an important role in different processes directly linked to immunity, such as the regulation of structural components, eicosanoid synthesis, formation of lipid peroxides, regulation of gene expression, apoptosis, alteration of antigen presentation or modulation of intestinal microbiota [1]. In mammals, FA administration enhances resistance to bacterial and viral pathogens by increasing survival and decreasing bacterial and viral loads [2–6]. The mechanisms by which FAs are able to activate the immune system in mammals include the enhancement of cytokine production in macrophages [7,8], regulation of apoptosis [9,10], and modulation of autophagy in hepatocytes [11]. It is becoming evident that the function and proportion of leukocyte subpopulations are greatly influenced by their intrinsic metabolic profile. In this sense, it has been shown that FA oxidation, together with glucose metabolism, plays a pivotal role in determining the cell phenotype of macrophages and T lymphocytes [12–17]. It has also been reported that neutrophils are in constant contact with FAs, which modulate cell functions through different mechanisms [18]. The outcome of the interaction, whether stimulation or inhibition of neutrophil recruitment, depends on different conditions, including the type and concentration of FAs [11,18].

Infectious diseases represent one of the major threats to fish aquaculture. The absence of commercially available vaccines or treatments for several diseases raises the importance of developing new strategies to prevent and/or combat infections. Moreover, if we also consider the increasing prevalence of antibiotic-resistant bacteria [19], then the development and use of alternative therapies are urgently needed to guarantee the sustainable development of fish aquaculture. Immunostimulation and nutritional approaches could represent an alternative way to ensure optimal fish growth performance and health [20].

It has been extensively explained that dietary manipulation can enhance the response to stress, injury and infection [21]. In addition to being considered a key source of energy [22], FAs can strongly influence these processes. Therefore, modulations in the level and composition of FAs could impact the immune system of fish. It is known that FA supplementation to the fish diet could be a promising approach in the culture of commercial fish species since FA-enriched feed has been shown to enhance the protection against several pathogens [20,23–29]. Fish fed with alternative oil sources showed enhanced phagocytic activity, higher antibody production and increased lysozyme activity

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[30].

Palmitic acid (PA) is a vegetable oil candidate for fish oil replacement in aquafeeds [31,32]. It is the most common FA found in animals, plants and microorganisms and a major component of the fruit from oil palms. It has been reported to possess pharmacological properties such as anticancer, anti-inflammatory or analgesic activities, and it also seems to modulate autophagy and NFκB signalling pathways in human cells [33–35]. Although few studies have focused on the properties of PA in the immune response of vertebrates other than mammals, a recent publication reported that the treatment of zebrafish larvae with PA induces nitrous oxide (NO) and reactive oxygen species (ROS) production and expression of pro-inflammatory cytokines [36]. An increase in the expression of pro-inflammatory cytokines was also observed in one-month-old zebrafish fed with various proportions of PA [36].

To further investigate the potential of this FA in aquaculture, we used the model species zebrafish and the viral pathogen spring viremia of carp virus (SVCV), a rhabdovirus associated with high mortalities in cyprinids. Because viral diseases are important concerns in fish aquaculture since they produce mortalities for which no treatments are available, the aim of the present study was to use different approaches *in vivo* and *in vitro* to examine the effect of the PA treatment on the survival and immunity of zebrafish. We found that the PA treatment reduced the mortality caused by SVCV and suppressed viral proliferation, likely by inhibiting autophagosome/lysosome fusion, the final step of autophagy. Because SVCV uses autophagy to replicate [37], our results suggest that PA reduces viral replication through the inhibition of autophagic flux.

2. Materials and methods

2.1. Fish, cell line, virus

Embryos and larvae from wild-type zebrafish were obtained from our experimental facility, where zebrafish are maintained following established protocols [38,39] (see http://zfin.org/zf_info/zfbook/zfbk.html). The transgenic lines Tg GFP-Lc3 (CMV:EGFP-map1lc3b) zf155 [40] and Tg Mpo:GFP (BAC-zC91B8) [41] were previously described. Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number ES360570202001/16/FUN01/PAT.05/tipoE/BNG.

Fibroblast-like ZF4 cells, derived from zebrafish embryos (ATCC CRL-2050) [42], were cultured in Dulbecco's modified Eagle's medium (DMEM/F12, Gibco) supplemented with 2% penicillin and streptomycin (InvivoGen) and 10% foetal bovine serum (FBS) at 27 °C.

The rhabdovirus spring viremia of carp virus (SVCV isolate 56/70) was propagated in ZF4 cells at 22 °C and titrated in 96-well plates. The $TCID_{50}$/ml was calculated according to the Reed and Muench method [43].

2.2. Reagents

Palmitic acid was obtained from Sigma-Aldrich (P0500) and dissolved in pre-heated 0.1 N NaOH at 60 °C as reported previously [36,44]. Two 10 mM stock solutions were prepared: a solution for the larvae treatment, which was generated by conjugating PA with a pre-warmed fatty acid-free BSA solution (Sigma-Aldrich, A7030) and a solution for the cell culture, which consisted of DMEM/F12 containing 12% (w/v) BSA. The autophagy inhibitors 3-methyladenine (3 MA) and chloroquine (CQ) were purchased from Sigma-Aldrich (#M9281 and #C6628, respectively) as was the autophagy activator rapamycin (RAPA; #R0395).

2.3. Treatment assays in vivo

2.3.1. Survival experiment and sampling procedures

At day 3 post-fertilization (dpf), wild-type zebrafish larvae were transferred to 6-well plates and maintained in 6 ml of filtered egg water (60 mg/ml sea salts in distilled water). Larvae were incubated at 27 °C in the presence of PA (1 mM) [36] for 24 h or in control medium containing 0.1 N NaOH and BSA without PA. Thereafter, the fish were rinsed in egg water three times, and one-half of the larvae from each treatment were infected with SVCV (4.3×10^5 $TCID_{50}$/ml) by bath immersion for 24 h. Mortality was assessed for 8 days post-infection (dpi) using three biological replicates composed of 10 larvae each. This experiment was conducted three times, and results from the replications were obtained. For the gene expression experiments, the same approach was used, and at 24 h post-infection (hpi), five or six pools (4–5 larvae/pool) were sampled and stored at −80 °C until RNA isolation.

2.3.2. Visualization of Lc3 abundance and neutrophil count

Transgenic GFP-Lc3 and Mpo:GFP larvae were used for the visualization of Lc3 abundance and for the neutrophil count, respectively. The experiment was conducted as in the previous section, but at 24 hpi, the larvae were anaesthetized by adding two drops of a 0.05% MS-222 solution to a Petri dish with a volume of 10 ml of egg water. Then, images of the whole larvae were taken using a Nikon AZ100 fluorescence microscope. To discard the potential autofluorescence of this FA, PA-treated wild-type zebrafish were also observed under the fluorescence microscope, and no positive fluorescence signal was obtained (data not shown).

2.4. Treatment assays in vitro

2.4.1. Viral titre estimation

The SVCV infectivity *in vitro* was evaluated by two different methods. In the first method, ZF4 cells were seeded on 96-well plates, and when the cells were at 70–80% confluence, the medium was removed and replaced by DMEM with penicillin/streptomycin and 2% FBS in the presence or absence of PA (1 mM), after confirming that this concentration did not affect cell viability. Seven 10-fold serial dilutions of SVCV (higher concentration: 4.3×10^5 $TCID_{50}$/ml) in DMEM with penicillin/streptomycin and 2% FBS were used for the viral titration in triplicate following the Reed and Muench method [43]. Uninfected controls were also included, and the plates were incubated at 27 °C. The evolution of the cytopathic effect (CPE) was checked every day using a Nikon Eclipse TS100 inverted microscope. To obtain replication results, this experiment was conducted three times.

The infectivity of SVCV *in vitro* was also evaluated by quantitative PCR (qPCR) detection of the SVCV N gene. ZF4 cells were seeded as described above and treated with 1 mM PA, 5 μM RAPA, or 1 mM PA and 5 μM RAPA and incubated with SVCV (4.3×10^3 $TCID_{50}$/ml). At 24 h post-infection, the medium was removed, and the cells were washed twice with PBS, collected (4 biological replicates) and stored at −80 °C until RNA isolation.

2.4.2. Cellular apoptosis

The ZF4 cells were seeded on 96-well plates, and when the cells were at 70–80% confluence, the medium was removed and replaced by DMEM with penicillin/streptomycin and 2% FBS containing 1 mM PA, SVCV (4.3×10^5 $TCID_{50}$/mL), or PA and SVCV. Uninfected controls were also included. Then, the cells were incubated at 27 °C, and after 3 h, the cells were centrifuged and resuspended in 100 μL of binding buffer (BB1X). Then, 5 μl of annexin V (Invitrogen) and 5 μl of 7-aminoactinomycin (7-AAD) (BD Pharmingen) were added to the cell suspensions. Samples were incubated for 15 min at room temperature in the dark and analysed by flow cytometry to study the putative differential level of cell death between the ZF4 cells treated with PA and those not treated with PA. Data acquisition was performed using a FACS Calibur flow cytometer (Becton and Dickinson), and analyses were carried out using CellQuest software (Becton and Dickinson).

2.4.3. Western blot analysis

To analyse the conversion of Lc3-I to Lc3-II as a means to estimate the abundance of autophagic-related structures, the ZF4 cells were seeded onto 24-well plates, and when they reached 70–80% confluence, the medium was removed and replaced by DMEM with penicillin/streptomycin and 2% FBS alone (control) or in combination with 1 mM PA, SVCV (4.3×10^5 $TCID_{50}$/ml) or PA and SVCV. After 24 h of incubation, the culture medium was removed, and the cells from the monolayers were resuspended in 150 µl of PBS with 2% Triton X-100 (Sigma-Aldrich), 1% of protease inhibitor cocktail (Sigma-Aldrich, #P8340) and a 10% of phosphatase inhibitor cocktail (Thermo Fisher, #78420). The cells were maintained on ice during the whole process to prevent protein denaturation. Homogenates were centrifuged at 14,000 rpm for 20 min at 4 °C, and the resulting supernatants were recovered. A total of 20 µg of protein was mixed with 1 × NuPAGE LDS sample buffer (Invitrogen) and resolved in a 4–20% Mini-PROTEAN TGX gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked for 2 h with 3% (w/v) bovine serum albumin (BSA) in TBST buffer (20-mM Tris, 0.5-M NaCl, and 0.1% Tween 20) and incubated for 1 h with a rabbit polyclonal anti-LC3A/B antibody (Cell Signalling Technology, #4108; diluted 1:500 in 1% BSA-TBST buffer) and a mouse monoclonal anti-actin antibody (Chemicon, #MAB1501; diluted 1:5000 in 1% BSA-TBST buffer) at room temperature. After it was washed, the membrane was incubated with a goat anti-rabbit IgG-HRP secondary antibody (Sigma-Aldrich, #A6154; diluted 1:6000) and detected by chemiluminescence with Luminata™ Forte Western HRP substrate (Millipore) and visualized with a ChemiDoc XRS Plus system (Bio-Rad).

2.4.4. Immunofluorescence detection of Lc3b

The ZF4 cells were seeded onto 24-well plates with 12-mm glass coverslips, and when the cells reached 70–80% confluence, the medium was removed and replaced by DMEM with penicillin/streptomycin and 2% FBS containing 1 mM PA, SVCV (4.3×10^5 $TCID_{50}$/mL) or PA and SVCV. Uninfected controls were also included. The plates were incubated at 27 °C, and after 24 h, the medium was removed, and the cells were fixed with 2% paraformaldehyde for 10 min at 4 °C. After they were washed, the cells were blocked by incubating them for 1 h in PBS with 0.1% saponin (Sigma-Aldrich) and 2% BSA (Sigma-Aldrich). Then, the cells were incubated overnight at 4 °C with a mouse anti-LC3B monoclonal antibody (Nanotools; #0231–100/LC3-5F10) (1:20) in staining buffer (PBS with 0.1% saponin and 0.1% BSA). The cells were then washed and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:1000) for 1 h at RT. All samples were stained with a 4′-6-diamidino-2-phenylindole (DAPI) solution (Molecular Probes-Life Technologies) for nuclear localization and with a phalloidin solution (Sigma-Aldrich) for actin filament localization. Prepared samples were mounted using ProLong Antifade reagent (Life Technologies). Confocal images were captured with a TSC SPE confocal microscope (Leica) using LAS AF software (Leica). The Lc3b intensity was measured using ImageJ software [45].

2.4.5. Lc3b and Lamp1 co-localization

The ZF4 cells were seeded onto 24-well plates with 12-mm glass coverslips in the absence of serum for inducing starving (pro-autophagic stimulus), and after 15 h, the medium was removed and replaced by DMEM with penicillin/streptomycin containing 1 mM PA, 5 µM RAPA or 1 µM CQ. Untreated controls were also included. The plates were incubated at 27 °C and, after 2.5 h, the cells were fixed and immuno-stained for Lc3b and Lamp1 detection following the protocol described in section 2.4.4. For Lc3b puncta identification, the mouse anti-LC3B monoclonal antibody (Nanotools; #0231–100/LC3-5F10; 1:20) and the secondary antibody, Alexa Fluor 635 goat anti-mouse IgG (1:1000), were used. For Lamp1, a rabbit polyclonal anti-Lamp1 (Abcam; #ab24170; 1:500) and the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (1:1000), were utilized. Confocal images were captured using a TSC SPE confocal microscope (Leica) using LAS AF software (Leica).

2.4.6. Lysosome detection

To visualize l the lysosomes, the ZF4 cells were seeded onto 24-well plates with 12-mm glass coverslips, and when they reached 70–80% confluence, the medium was removed and replaced by DMEM with penicillin/streptomycin and 10% FBS (control), or medium containing 1 mM PA, 5 µM RAPA, 5 mM 3 MA or 1 µM CQ. After 2.5 and 24 h, two sets of cells, respectively, were washed and incubated with 1 µM of LysoSensor Blue DND-167 reagent (Molecular Probes-Life Technologies), which is used to stain the acidic lysosomal vesicles in live cells, and incubated for 30 min in the dark. Preparations were mounted using ProLong Antifade reagent (Life Technologies). Confocal images were captured using a TSC SPE confocal microscope (Leica) using LAS AF software (Leica).

2.5. RNA extraction, cDNA synthesis and quantitative PCR analysis

Total RNA was extracted from zebrafish larvae and ZF4 cells using the Maxwell 16 LEV SimplyRNA Tissue kit (Promega) with the automated Maxwell 16 Instrument, in accordance with instructions provided by the manufacturer. cDNA synthesis was performed with the NZY First-Strand cDNA synthesis kit (NZYtech) using 500 ng of RNA.

The quantitative analysis of gene expression was determined using a 7300 Real-Time PCR System (Applied Biosystems). The cDNA was amplified using specific primers for the *beclin1*, *ambra1a*, *atg5*, *gabarap*, *ifnphi1*, *ifnphi2* and SVCV N genes, which were previously described in different publications [46–49]. The primer sequences are shown in Table 1. The primer amplification efficiency was calculated according to the threshold cycle (CT) slope method [50]. Analyses were performed with 1 µl of cDNA using the SYBR GREEN PCR Master Mix (Applied Biosystems) in a total PCR volume of 25 µl that contained each primer (10 µM). The thermal cycling was initiated with a denaturation step (95 °C, 10 min) followed by 40 cycles of a denaturation step (95 °C, 15 s) and one hybridization-elongation step (60 °C, 1 min). mRNA levels of the target genes were normalized with the reference gene *18s ribosomal RNA*, which was stably expressed in this experiment. Expression levels were calculated according to the Pfaffl method [50].

Table 1
Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by quantitative PCR (qPCR).

Gene	Forward primer	Reverse primer
18s ribosomal RNA	ACCACCCACAGAATCGAGAAA	GCCTGCGGCTTAATTTGACT
SVCV N gene	TGAGGTGAGTGCTGAGGATG	CCATCAGCAAAGTCCGGTAT
ifnphi1	GAGCACATGAACTCGGTGAA	TGCGTATCTTGCCACACATT
ifnphi2	CCTCTTTGCCAACGACAGTT	CGGTTCCTTGAGCTCTCATC
beclin1	GATCATGCAATGGTGGCTTTC	CCTCCTGTGTCCTCAATCTTT
ambra1a	TCTTTCGAGAAATGGCACCT	CTCTCTGCGTTAGGGACAGG
atg5	AGAGAGGCAGAACCCTACTATC	CCTCGTGTTCAAACCACATTTC
gabarap	GTCTGACCTCACAGTTGGGC	TCCTGGTAGAGCAGTCCCAT

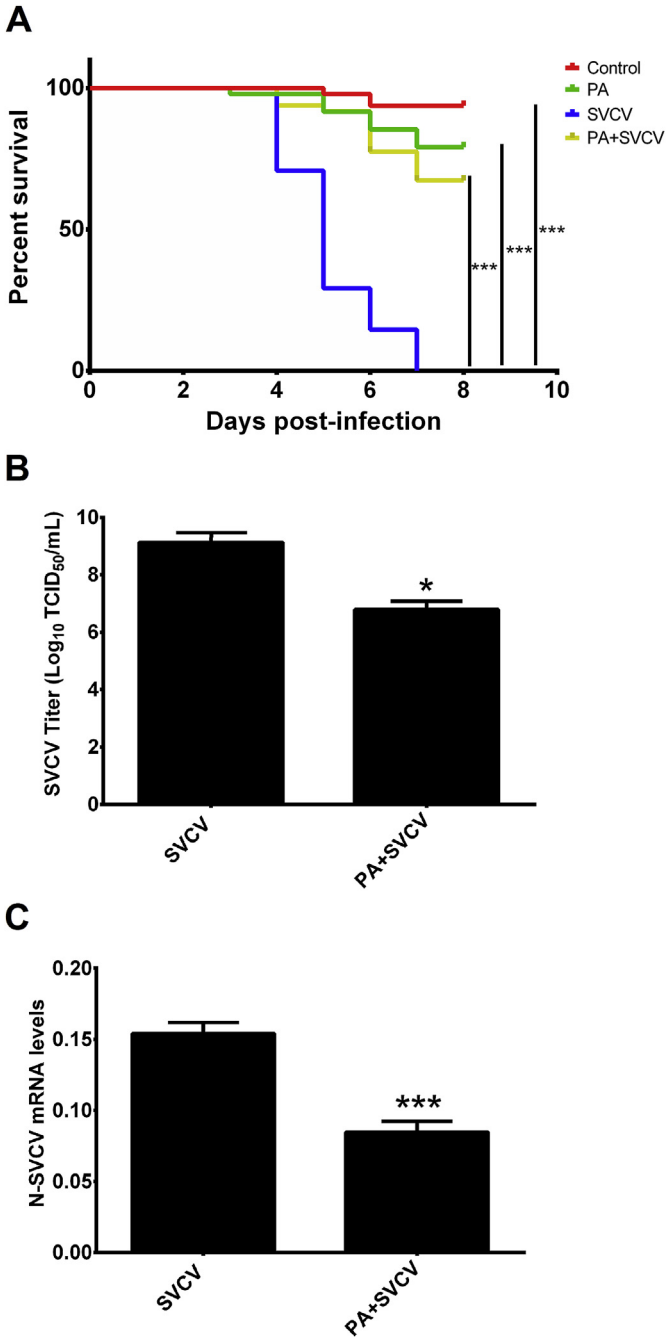

Fig. 1. Effect of PA treatment on the survival of zebrafish larvae after an SVCV challenge (A) and antiviral effect of PA in the ZF4 cells (B, C). (A) Kaplan-Meier survival curves of zebrafish larvae. Data were analysed for statistical significance with a log-rank (Mantel-Cox) test. The graphs represent the means of 3 independent experiments, and the data are presented as the means ± SEM. (B) Effect of PA treatment in the viral titre of ZF4 cells infected with SVCV and calculated following the Reed and Muench method. The graphs represent the means of 3 independent experiments, and the data are presented as the means ± SEM. (C) Detection of the SVCV nucleoprotein (N) gene in ZF4 cells treated or not with PA at 24 hpi. The graph represents the means ± SEM of 5 independent biological replicates. Significant differences are displayed as ***($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

2.6. Statistics

Comparisons among groups were made using Student's t-test (paired comparisons) or one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test, and the differences were considered statistically significant at $p < 0.05$. When necessary, the data were log-transformed to fulfil the conditions of the analysis of variance.

In survival experiments, Kaplan-Meier cumulative survival curves were analysed for statistical significance with a log-rank (Mantel-Cox) test. Significant differences are displayed as *** ($p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

3. Results

3.1. Palmitic acid treatment enhances zebrafish survival and reduces viral replication after an SVCV challenge

We first evaluated the capacity of PA to protect against an SVCV infection *in vivo* (Fig. 1A). Mortality started at 4 dpi and, since that day, we observed that treatment with PA increased the resistance of zebrafish larvae against viral infection. At the end of the experiment, the survival percentage was 0% for the untreated fish and 67.3% for the PA-treated fish. There were no significant differences in the survival of the control and the PA-treated larvae without infection.

We also analysed the antiviral effect of PA in ZF4 monolayers. A reduction of 2-logarithm (log) in the virus titre (significant differences $0.01 < p < 0.05$) was observed when the cells were treated with PA (Fig. 1B). Differences in the replication of SVCV in the ZF4 cells were also found by analysing the expression of the SVCV N gene at 24 hpi (Fig. 1C). A significant ($p < 0.001$) reduction in the detection of the N gene was observed in the cells treated with PA compared to the gene level detected in cells threated with only the virus.

3.2. Palmitic acid does not increase apoptosis, type I interferon expression or neutrophil count during SVCV infection

We next studied the potential mechanisms that could be involved in the antiviral effect of PA against SVCV (Fig. 2). When ZF4 cells were exposed to 1 mM PA, a significant increase in the percentage of apoptotic cells was observed compared to that observed in the untreated and uninfected cells ($p < 0.001$). SVCV infection also elevated apoptosis levels in both the untreated and PA-treated cells ($p < 0.001$). However, although the administration of PA increased apoptosis, per se, it did not influence the percentage of apoptotic cells in the infected ZF4 cells (Fig. 2A).

To further determine whether the antiviral protection provided by PA was due to the potentiation of the type I IFN response, the expression of *ifnphi1* and *ifnphi2* was analysed in zebrafish larvae. As expected, SVCV infection induced the expression of the *ifnphi1* gene ($p < 0.001$) compared to that of the uninfected control. However, no significant differences were noticed in zebrafish larvae treated with PA or with PA and SVCV compared to the control (Fig. 2B). Indeed, PA downregulated the *ifnphi1* expression induced by SVCV. On the other hand, *ifnphi2* expression was not affected by any of the treatments (Fig. 2C).

Finally, to determine whether neutrophil proliferation could be a mechanism involved in resistance, we confirmed that the neutrophil number in the Mpo:GFP zebrafish larvae was not affected by PA and/or SVCV (Fig. 2D and E).

3.3. Palmitic acid alters the autophagic process

Autophagy has been described as an efficient antiviral mechanism in response to certain viral infections [51–54]; however, at the same time, it is known that SVCV uses autophagy to facilitate its own replication [37]. Therefore, we investigated whether PA could mediate the clearance of SVCV via autophagy induction (Fig. 3). The use of the transgenic zebrafish line GFP-Lc3 enabled us to observe the expression of the central component of autophagy, Lc3, in live larvae (Fig. 3C). The results from the analysis of the images revealed that, whereas no significant modulation was observed in the larvae treated with SVCV

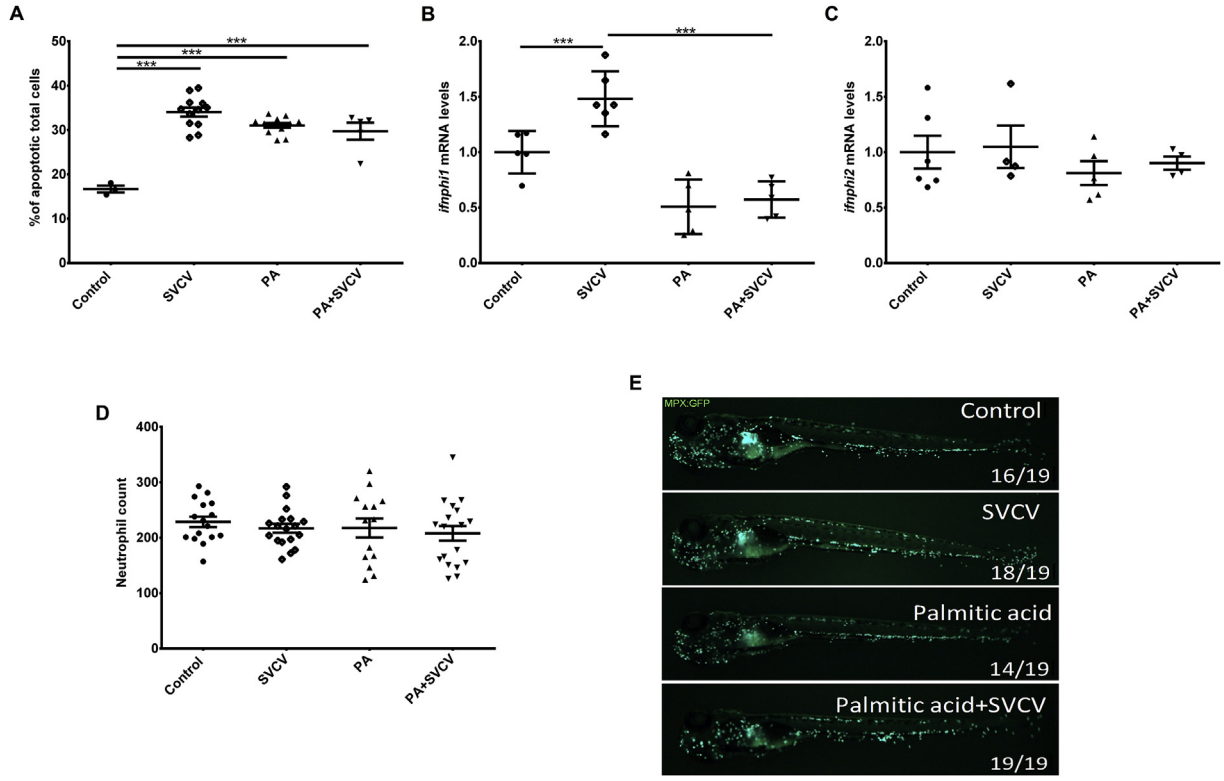

Fig. 2. Measurement of different immune parameters after PA treatment. (A) Effect of PA and SVCV infection in the apoptosis of ZF4 cells measured by flow cytometry. Data represent the mean of four independent experiments ± SEM. (B, C) Relative mRNA abundance of *ifnphi1* (B) and *ifnphi2* (C) in pools of 4–5 zebrafish larvae treated or not with PA in the absence or presence of SVCV infection. The data represent the mean ± SEM of 5–6 measurements. (D, E) Neutrophil count in the transgenic Mpo:GFP zebrafish larvae treated with PA for 24 h and then infected or not with SVCV. The numbers in the images represent the animals with the shown phenotype per total analysed larvae. The neutrophil count is presented as the mean ± SEM of the analysed fish by treatment. Comparisons among groups were made using one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

alone, the Lc3-associated fluorescence significantly increased in the body muscle mass ($p < 0.001$) (Fig. 3A) and liver ($0.001 < p < 0.01$) (Fig. 3B) of the larvae treated with PA compared to the levels observed in the untreated control individuals. The higher level of Lc3 signal was also maintained in the larvae treated with PA + SVCV, especially in the body muscle mass ($p < 0.001$) (Fig. 3A).

To further investigate the effect of PA on the autophagy mechanism, the ZF4 cells were incubated with FA and/or infected with SVCV for 24 h to determine the autophagy levels as indicated by cellular Lc3 distribution (Fig. 4A). According to the results *in vivo*, the PA treatment increased the accumulation of Lc3-positive autophagosomes in the absence or presence of SVCV ($p < 0.001$) (Fig. 4B). To thoroughly assess the autophagy through Lc3, western blotting was performed from lysates of ZF4 cells at 24 h post-treatment and/or post-infection (Fig. 4C and D). We studied the extent of autophagy by analysing Lc3-I/Lc3-II conversion [55]. The Lc3-II/Lc3-I ratio significantly increased ($p < 0.05$) in the cells treated with PA ± SVCV compared to the control and SVCV groups (Fig. 4C and D).

Nevertheless, because certain autophagy inhibitors also increase the detection of autophagosomes by blocking autophagosome/lysosome fusion, thereby suppressing their degradation, we used the autophagy inducer RAPA to evaluate whether autophagy induction effectively reduces SVCV replication. We used RAPA and PA alone and in combination to analyse the replication of SVCV in the ZF4 cells (Fig. 4E). Whereas the use of PA reduced the level of detected virus compared to levels in untreated but SVCV-infected cells ($0.01 < p < 0.05$), RAPA increased viral replication ($0.01 < p < 0.05$). Moreover, the use of PA and RAPA showed an intermediate viral proliferation level.

3.4. Palmitic acid inhibits autophagosome/lysosome fusion

Cells activate autophagy under starving conditions, and RAPA also contributes to the activation of this process. Whereas RAPA increased the co-localization of Lc3b and Lamp1 in starved ZF4 cells, the Lc3b- and Lamp1-positive puncta were reduced by the administration of PA (Fig. 5), indicating impaired autophagosome/lysosome fusion. Interestingly, although CQ is known as an autophagy inhibitor that prevents autophagosome/lysosome fusion, we also observed a reduction in Lamp1 fluorescence after this treatment (Fig. 5).

3.5. Palmitic acid increases the number of lysosomes in ZF4 cells

When ZF4 cells were treated for 2.5 h with PA and different autophagy modulators, we observed that PA significantly increased the number of LysoSensor-positive dots per cells compared to the number observed from untreated cells ($p < 0.001$) (Fig. 6A and B), and a similar pattern was observed for the autophagy inhibitor CQ ($p < 0.001$) (Fig. 6A and B). Although CQ-treated cells showed a reduction in Lamp1 fluorescence, the presence of lysosomes was confirmed, which could be indicative of Lamp1 modulation. On the other hand, the autophagy inducer RAPA and the inhibitor 3 MA did not significantly affect the number of lysosomes (Fig. 6A and B). Therefore, it seems that PA exerts a similar effect to that induced by CQ. PA showed a time-increase effect in ZF4 cells, with a significantly higher number of lysosomes per cell at 24 h (Fig. 6C) compared to the number at 2.5 h (Fig. 6D).

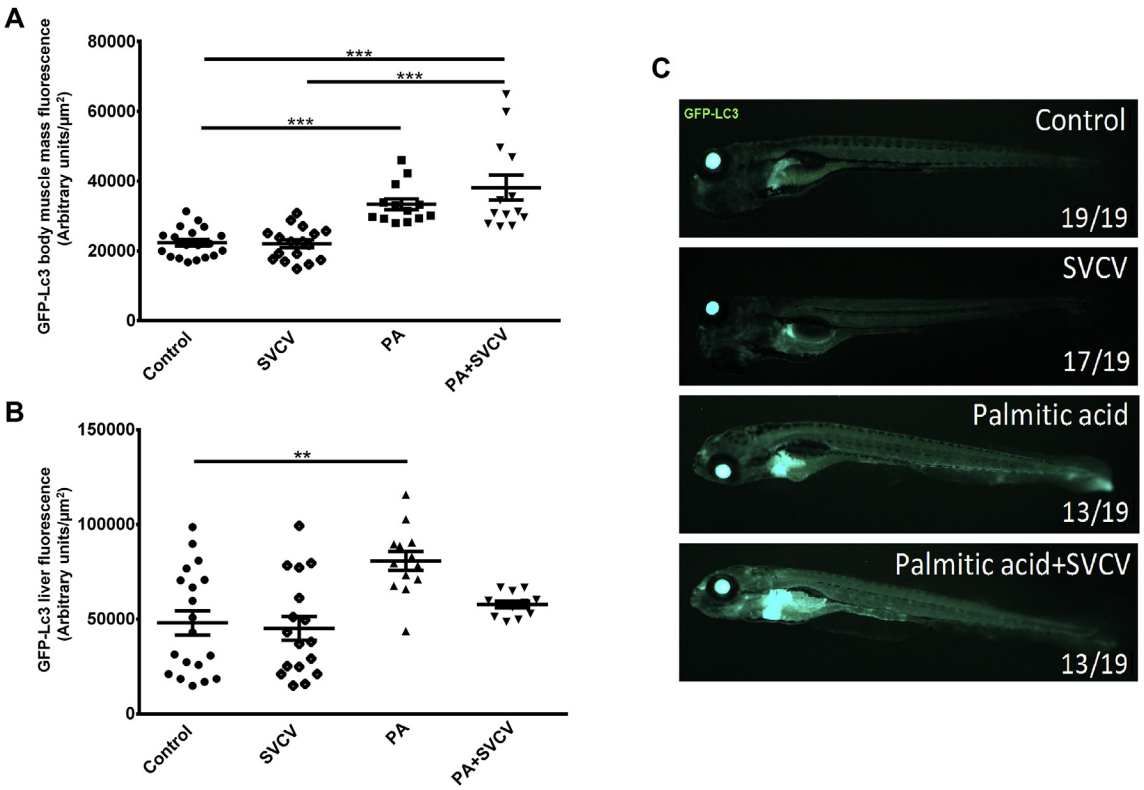

Fig. 3. Palmitic acid activates autophagy in zebrafish larvae. Zebrafish GFP-Lc3 transgenic embryos were treated with PA by bath immersion, and 24 h later, they were infected with SVCV for 24 h. The intensity of the GFP-Lc3 signal was measured in the body muscle mass (A) and liver (B). Numbers in the images represent the animals with the shown phenotype per the total number of animals analysed (C). The results are presented as the mean ± SEM of the analysed fish by treatment. Comparisons among groups were made using one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

3.6. Palmitic acid modulates the expression of autophagy-related genes in vivo

The expression of some autophagy-related genes was also evaluated in zebrafish larvae pre-treated with or without PA for 24 h and subsequently infected with SVCV or not (Fig. 7). In the absence of infection, the PA treatment increased the expression of *beclin1* (Fig. 7A) and *gabarap* (Fig. 7D). On the other hand, SVCV induced an increase in the expression of *atg5* compared to the untreated and uninfected control (Fig. 7C). Interestingly, the mRNA levels of *beclin1* (Fig. 7A) and *atg5* (Fig. 7C) were lower in the PA + SVCV group compared with the SVCV and PA groups, and *gabarap* (Fig. 7D) showed lower expression in the PA + SVCV individuals compared to the PA-treated group. Finally, no significant modulations were noted in the mRNA levels of *ambra1a* (Fig. 7B).

4. Discussion

In mammals, several FAs are involved in different immune functions and can induce changes in mechanisms with significant roles in inflammatory processes and in the resistance of the host against infectious microorganisms [1]. As in humans, the concept that better nutrition leads to improved health also applies to aquatic animals. In aquaculture, dietary lipids have been used to enhance resistance and survival against bacterial and viral infections and to regulate the innate response during infectious diseases [20,23–29]. It has been shown that high concentrations of PA have toxic effects on zebrafish embryos, likely due to the increase in the expression of pro-inflammatory cytokines [36]. However, it is practically unknown how non-toxic concentrations of FAs, such as PA, can affect immune parameters in zebrafish. In the present study, we assessed whether treatment with PA was able to ameliorate the resolution of SVCV infection in zebrafish. We demonstrated that treatment by bath immersion with 1 mM PA increases survival after SVCV infection in zebrafish larvae. Further, this increase in survival is in agreement with the *in vitro* assays in ZF4 cells treated with PA, which revealed a reduction in the viral titre and in the expression of the SVCV N gene, reflecting the antiviral effect of this FA at low concentrations. The protection obtained is comparable to that observed in other fish species against pathogens when they are feeding with FA-enriched diets [20,23–29]. Nevertheless, the mechanism explaining how PA inhibits viral infection has not yet been proposed.

One of the most plausible explanations for this antiviral activity could be the potentiation of the IFN system. Type I interferons (IFNs) are the main cytokines regulating the antiviral innate immune response in vertebrates [56]. IFNs induce the expression of IFN-stimulated genes (ISG), which encode proteins with direct antiviral activity [57]. However, we found that PA did not affect the expression of the type I IFNs *ifnphi1* and *ifnphi2*. Hence, the antiviral mechanism based on the induction of IFNs seems not to be involved in the protection against SVCV provided by the PA in zebrafish larvae.

Neutrophils are the most abundant type of circulating leukocyte in zebrafish and play a critical role in the immune response to infection [58,59]. However, neutrophils can have beneficial and detrimental effects [60], and transmigration of these cells through the vascular endothelial walls into inflamed tissues is a critical defence mechanism of the innate immune system against infection and chronic inflammatory diseases [61]. Therefore, we further analysed changes in the number of neutrophils in the transgenic line Tg Mpo:GFP larvae after PA treatment and found that this was not altered by FA. Our results are in agreement with studies in humans that showed that PA does not significantly alter chemotaxis and phagocytosis [62] and that this FA induces the production of significant amounts of ROS in the absence of any neutrophil

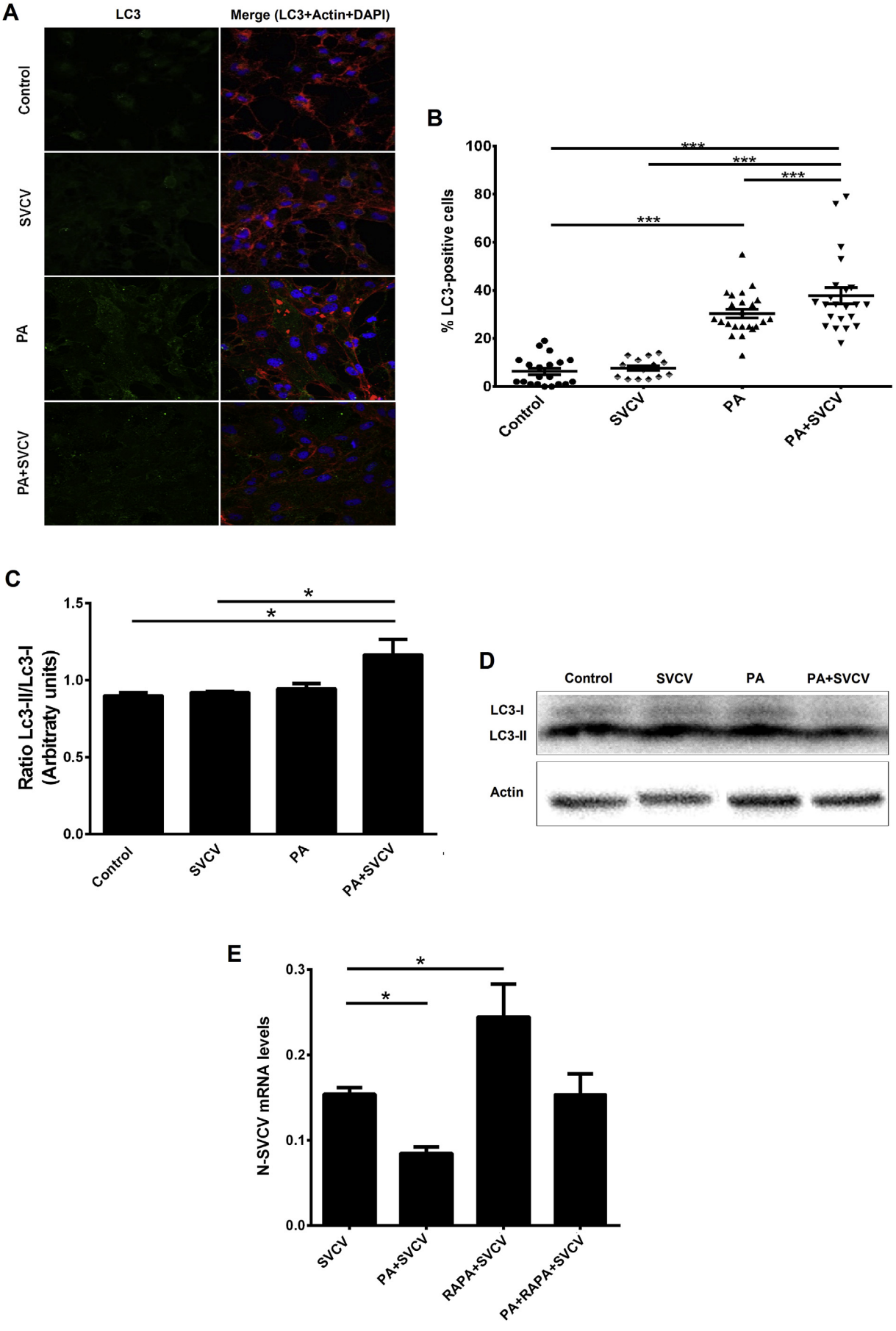

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Fig. 4. Palmitic acid increases the Lc3b signal in ZF4 cells. (A) The ZF4 cells were incubated with PA for 24 h and subsequently infected or not with SVCV. At 24 hpi, the cells were fixed and immuno-stained for Lc3b (green) and actin (red) detection. Nuclei were stained with DAPI (blue). Images were taken by confocal microscopy to determine the proportion of Lc3-positive cells. (B) The percentage of Lc3-positive ZF4 cells after different treatments. Data are presented as the mean ± SEM of four independent experiments. (C, D) Western blot analysis of the Lc3 in ZF4 cells after PA treatment and/or SVCV infection. Graphs represent the Lc3-II/Lc3-I ratio. The actin bands were detected using an anti-actin antibody as an internal control for protein load. Data are shown as the mean ± SEM of 4 independent experiments. (E) Detection of the SVCV N gene in ZF4 cells incubated with PA, RAPA or PA + RAPA for 24 h and then infected with SVCV. The graph represents the means ± SEM of 5 independent biological replicates. Comparisons among groups were made using Student's t-test (paired comparisons) or one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test, and the differences were considered statistically significant at $p < 0.05$. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stimulus [63].

Infections modulate divergent host immune responses, including apoptosis or programmed cell death [64,65]. It has been shown that PA also increases apoptosis [66,67]. Concordantly, we found that both PA and SVCV induce apoptosis, but the presence of PA did not increase the number of apoptotic cells in SVCV-infected cells. As in the case of the induction of IFNs mentioned above, the transmigration of neutrophils and apoptosis do not seem to be the mechanisms involved in the protection provided by PA against SVCV.

On the other hand, it is known that pathogen infections can induce autophagy, and this process may have evolved as a powerful tool of the cellular innate immunity to target and degrade incoming pathogens, such as bacteria or viruses [68]. Nevertheless, in the case of SVCV, it has been reported that this virus induces autophagy to promote its own replication [37]. In the present study, SVCV was not able to significantly affect the Lc3 signal both *in vivo* and *in vitro*, but zebrafish larvae and ZF4 cells treated with PA alone or in combination with SVCV exhibited higher levels of Lc3-positive signal. In ZF4 cells, the effects were even higher with the combination of PA and SVCV, which was also reflected in a higher Lc3-II/Lc3-I conversion ratio. Increases in Lc3 detection are usually misinterpreted as increased autophagy activation. However, the use of autophagy inhibitors, such as CQ and bafilomycin, increases the accumulation of autophagosomes and, accordingly, LC3 detection [69,70]. In order to better understand if PA is inducing or inhibiting autophagy, we analysed the effect of the autophagy inducer RAPA on viral replication, and we observed a greater SVCV detection when ZF4 cells were incubated with this drug, indicating that autophagy benefits the virus. The addition of PA to the RAPA-treated cells significantly reduced viral replication, which likely indicated that PA and RAPA mediate opposite effects during autophagy. The higher Lc3 signal detection in cells treated with both PA and SVCV is likely due to the cumulative effect of autophagy induction by the virus, but autophagy suppression by PA in the final steps of autophagosome processing, as was previously observed for the combination of RAPA and CQ [69].

Different publications reported opposite effects of PA in the autophagy mechanism. Some works claimed the pro-autophagic role of this FA [71–75], whereas a similar number of publications indicated its inhibitory activity during autophagic flux [76–82]. These differences could be due to the different cell types used, the PA concentrations and different exposure times, but could be due to misinterpreted results because of the complexity of autophagy. Most of the publications reporting an inhibitory role of PA in autophagy suggest that this FA inhibits autophagosome/lysosome fusion, as occurs with CQ [83]. Indeed, this is one of the main mechanisms proposed to explain PA-induced apoptosis [78,79].

To compare the effect of PA to that induced by CQ, we treated ZF4 cells with RAPA, CQ or PA, and the co-localization of Lc3b and the lysosome marker Lamp1 was analysed. As expected, RAPA increased the co-localization of both proteins, but in the case of CQ and PA, the Lc3b-Lamp1 puncta were almost inexistent. Nevertheless, for CQ, lower detection of Lamp1 was also observed. As this could be indicative of lysosome degradation, we stained live ZF4 cells with LysoSensor Blue DND-167 after treatment with RAPA, 3 MA, CQ or PA. LysoSensor is used to measure acidic lysosomal vesicles and becomes more fluorescent with acidic pH values. Whereas a low number of LysoSensor-positive cells were observed in the control, RAPA- and 3 MA-treated cells, a significant increase was found with CQ and PA, indicating that lysosomes remained acidic but accumulated in the cells. Therefore, both CQ and PA diminished autophagosome/lysosome fusion without apparently affecting the lysosome functionality or, at least, not the pH level. The mechanism by which PA inhibits the fusion of both structures remains controversial. Interestingly, 3 MA, which is also an inhibitor of autophagy by blocking the formation of autophagosomes [84–86], did not increase the accumulation of lysosomes.

Autophagy requires a core set of conserved proteins known as autophagy-related (Atg) proteins [87]. Atg proteins are involved in the

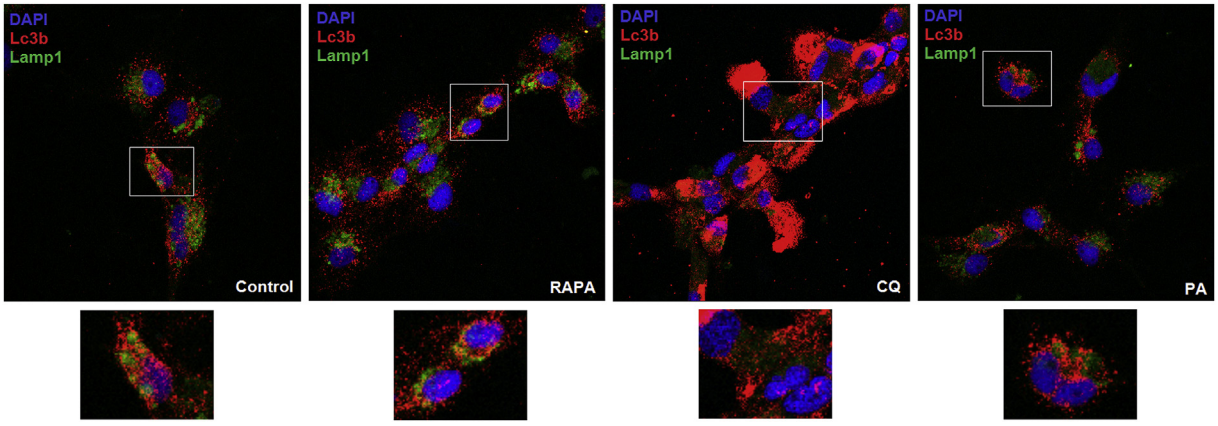

Fig. 5. Palmitic acid inhibits autophagosome-lysosome fusion. The ZF4 cells under conditions of starvation were incubated with PA, RAPA or CQ for 2.5 h. Untreated controls were also included. After the incubation period, the cells were fixed and immuno-stained for Lc3b (red) and Lamp1 (green) detection. Nuclei were stained with DAPI (blue). Images were taken by confocal microscopy to determine the prevalence of the co-localization of Lc3b and Lamp1. Representative images were selected to reflect the effect of the different compounds on the autophagic flux. Yellow/orange puncta indicate co-localization of Lc3b and Lamp1 (autophagic flux). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

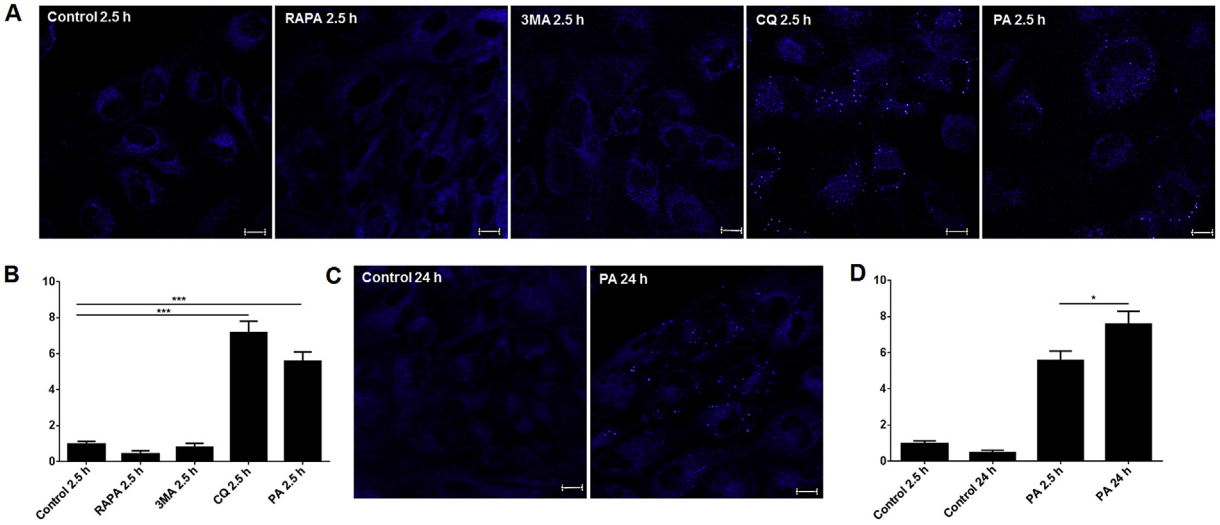

Fig. 6. Palmitic acid does not affect lysosome acidity and integrity but increases the number of lysosomes per cell. The ZF4 cells were incubated for 2.5 h with PA, 3 MA, RAPA or CQ. Untreated controls were also included. For PA and untreated control, the ZF4 cells were also incubated for 24 h. Live cells were stained with LysoSensor Blue DND-167 reagent for acidic lysosomal vesicle detection. Images were taken by confocal microscopy. (A, B) Compared to the respective controls, RAPA- and 3 MA-treated cells showed no significant differences in the number of lysosomes after 2.5 h. CQ and PA induced a significant increase in the number of lysosomes per cell compared to the other treatments. (C, D) The accumulation of lysosomes induced by PA persisted after 24 h and was significantly higher compared to the cells stimulated for 2.5 h. Data are shown as the mean ± SEM of the number of lysosomes per cell counted in 8–10 images per treatment. Comparisons among groups were made using one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

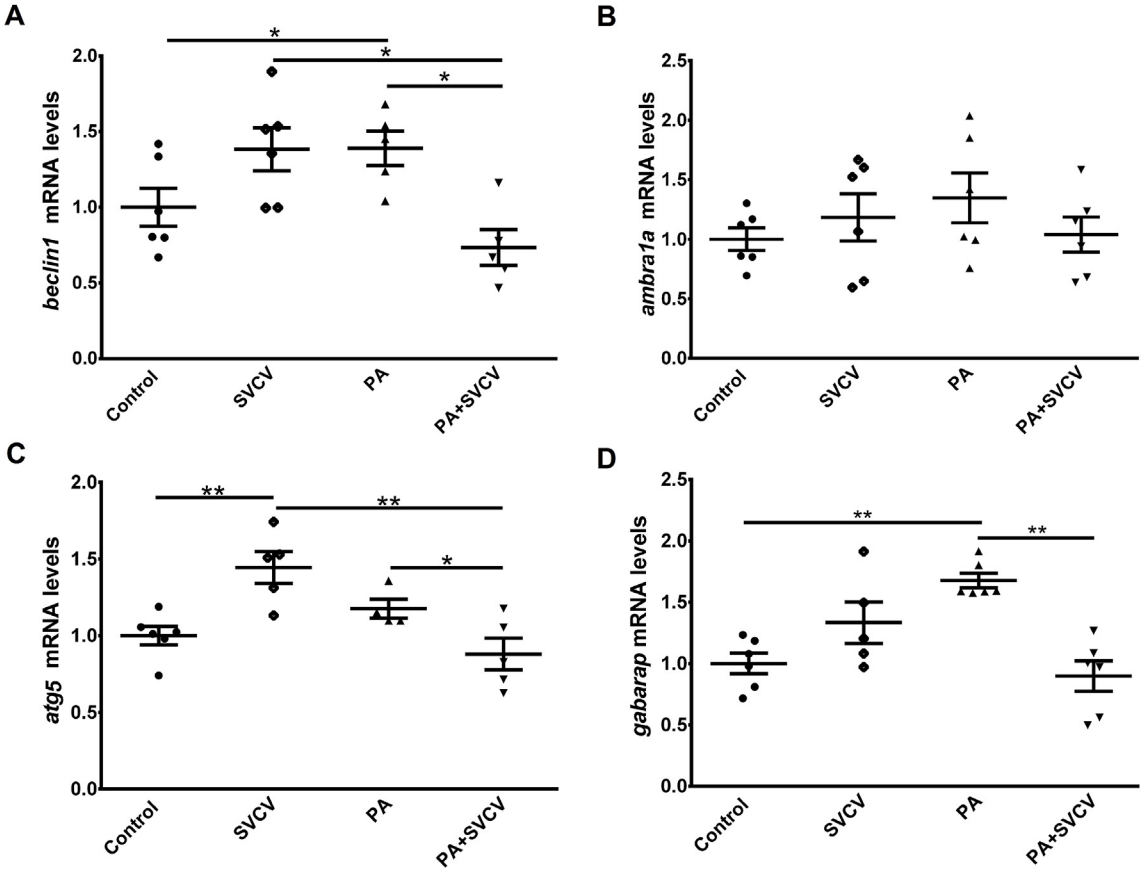

Fig. 7. Relative mRNA abundance of *beclin1* (A), *ambra1a* (B), *atg5* (C), and *gabarap* (D) in pools of 4–5 zebrafish larvae treated or not with PA and then infected with SVCV. Data represent the mean ± SEM of 5–6 measurements. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

induction of the phagophore, its expansion to an autophagosome, and finally the latter's fusion with the lysosome [87–89]. Because autophagy is tightly regulated and balanced by distinct regulatory pathways, to further understand whether PA also affects the expression of the autophagy components, we analysed the mRNA levels of some of these Atg proteins. In our results, *beclin1,* a key protein that plays an important role in a critical step of the autophagic process, namely, autophagosome formation, and *gabarap*, crucial for autophagosome formation and sequestration of cytosolic cargo into double-membrane vesicles, showed overexpression after PA treatment. Interestingly, when the zebrafish larvae were treated with PA and infected with SVCV, no modulations were observed in the expression of autophagy-related genes. This finding could indicate that the suppression of autophagic flux by PA could be interpreted as a state of deficiency in the degradative system and, as a consequence, the cells try to compensate by upregulating the expression of autophagy-related genes. On the other hand, because SVCV activates autophagy, the effects of PA on gene expression could be counteracted.

In conclusion, in the present study, we describe, for the first time in fish, the potential antiviral mechanism of PA treatment. Although the administration of PA at high concentrations usually induces toxic effects, low concentrations of this FA could benefit the resolution of viral infections. Our results showed that treatment with PA reduces the mortality caused by SVCV infection both *in vivo* and *in vitro*. Although some of the main antiviral mechanisms were studied in this work, only the inhibition of the autophagic flux seems to be involved in the protection provided by PA. Therefore, PA could be a useful therapeutic compound to prevent viral outbreaks in aquacultured fish. Nevertheless, the autophagy suppression caused by PA could also generate collateral detrimental effects in cells, such as apoptosis or senescence. Therefore, the use of this FA in aquaculture should be carefully considered.

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